Identification and characterisation of anti-platelet antibodies in ITP patients

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To

My Wife & children

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Abstract

The autoimmune disease known as autoimmune thrombocytopenic purpura (ITP) is clinically defined by a low numbers of platelets in the circulation blood. Anti-platelet antibodies bind to glycoprotein molecules on the membranes of platelets and result in their dysfunction and destruction. Despite a growing body of information about ITP, it is difficult to isolate and characterise anti-platelet antibodies, because only limited monoclonal antibodies are available from ITP patients.

This study used a phage display system to recognise Fab anti-platelet antibodies. Anti-platelet Fab-expressing phage was isolated by sequential panning of an ITP Fab library against normal non-ITP platelets. After isolation, the anti-platelet Fab-expressing phage was characterised by ELISA and Western blotting.

The Fab-bearing phage pool obtained from five rounds of panning was analysed in order to determine its anti-platelet reactivity. Of the phage colonies obtained, 100 colonies of different sizes were randomly selected for reaction with whole platelets, using M13 phage as a negative control. 12 colonies of them had strong reactions against the *whole* platelet preparation, but only four colonies showed substantial reactivity against the *lysed* platelet preparation (lysate). Colony S7 showed highest the greatest degree of binding to both the lysate and the whole platelet preparation. The specificity of the four colonies (S2, S7, S8 and S9) that had strong positive reactions against platelet antigens was determined for the glycoprotein component GP IIb/IIIa.

Further characterisation of the proteins in the lysate preparation was carried out using blotting techniques. The protein content of the four Fab-bearing phage colonies was quantified under the non-reducing conditions of Western blotting to evaluate their ability to recognise platelet antigens. Three of the four colonies showed three bands representing proteins with different molecular weights. Each of these three colonies had one band that corresponded to a protein of molecular weight 92 kD. The fourth colony showed only a single band, but this band also corresponded to a 92-kD protein.

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Abbreviations

ACD	Acid citrate dextrose solution
ADP	Adenosine diphosphate
AMLR	Autologous mixed lymphocyte reaction
APC	Antigen presenting cell
BSA	Bovine serum albumin
C	Complement system
cDNA	Complementary DNA
CDR	Complementarity determining region
CM	Trilaminar unit membrane
CMI	Cell mediated immunity
D	Diversity
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Exterior coat
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallisable region
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP-140	Granule membrane glycoprotein of 140kd
GP	Glycoprotein
HPA	Human platelet antigen
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HRP	Horseradish peroxidase
IFNδ	Interferon gamma
ITP	Idiopathic thrombocytopenic purpura
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J	Joining
kD	Kilo Dalton
LB	Luria broth
LIBS	Ligand-induced binding sites
MACE	Modified Antigen Capture ELISA
MAIPA	Monoclonal Antibody Immobilisation of Platelet Antigen
M-CSF	Macrophage colony- stimulating factor
MHC	Major histocampatibility complex
MIF	Migration inhibition factor
MPV	Mean platelet volume
mRNA	Messenger RNA
MW	Molecular weight
Neg	Negative
OCS	Open canalicular system
OD	Optical density
OPD	*O*-Phenylenediamine dihydrochloride
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGR	Platelet-derived growth factor
PEG	Polyethylene glycol
PIFT	Platelet Immunofluorescence Tests
PLA	Platelet antigen

PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PRAT	Platelet Radioactive Antiglobulin Test
RGD	Arginin-glycine-aspargine
RNA	Ribonucleic acid
RP	Reticulated platelet
SB	Super broth
SE	Standard error
TCR	T-cell receptor
TGF-β1	Transforming growth factor
Th	T helper cell
TNFα	Tumour necrosis factor alpha
TPO	Thrombopoietin
TXA2	Thromboxane A2
V	Variable
VWF	Von Willebrand factor

CONTENTS

INTRODUCTION

Idiopathic (immune) thrombocytopenic purpura (ITP) is an autoimmune disease that involves the development of autoantibodies against platelet cells in the blood, which play an essential role in the haemostatic control of bleeding. ITP is characterised by the premature immune destruction of platelets, with production of autoantibodies against platelets, specifically against the glycoprotein complexes in the platelet membrane, such as GP IIb/IIIa or GP Ib/IX. The glycoprotein complexes are physiological receptors that mediate interactions between platelets and vascular subendothelium in injured blood vessels. By their adhesion and aggregation activities, the platelets arrest haemorrhaging from lesions in the blood vessel wall, and any disruption to their membrane glycoproteins leads to platelet dysfunction and destruction, and causes the bleeding disorders seen in ITP. This study aimed to isolate autoantibodies made against the platelet glycoproteins using platelets from healthy volunteers, to determine their specificity and further elucidate their effects on platelet function. As there is no accurate diagnostic test for ITP, new evidence about these autoantibodies may be of great value in the future.

SECTION I: IDIOPATHIC (IMMUNE) THROMBOCYTOPENIC PURPURA (ITP)

I–1 Features and History of ITP

The main clinical sign of ITP is purpura. Purpura describes a skin disorder involving patches of discoloration that do not blanch (disappear) when pressure is applied. They are caused by bleeding beneath the skin because of a failure in some part of the blood clotting mechanism. The first descriptions of purpura date back to ancient Greece (Jones 1933), and in the 18th century a young woman with spontaneous petechiae and haemorrhages from her nose and mouth was described in England (Beutler 1995). The patches on the skin can be purple or red in colour and usually measure up to 1 cm across (patches of less than 3 mm across are known as petechiae and those of over 1 cm are known as ecchymoses). Purpura is not a disease, but a medical sign with several possible underlying mechanisms. For example, it might be caused by a vascular disorder or coagulation disorder, or meningococcal disease or a psychogenic problem.

This study is concerned with a coagulation disorder that involves an underlying platelet disorder, namely thrombocytopenic purpura. The term "thrombocytopenia" simply refers to a low level of platelets (thrombocytes) in the blood circulation of any cause. An association between the presence of purpura in thrombocytopenic patients and platelets was made by Simian and Zimmermann in the 19th century, after the observation of "small colourless bodies" in their blood. These findings added to scientific advances at that time about the processes of fibrin clots, blood coagulation and thrombosis (Bizzozero 1881), and the

function of platelets and their role in the aetiology of thrombocytopenic purpura gradually became clear (Howell 1890; Wright 1906).

The bleeding tendency in ITP is caused by a platelet disorder (White 1994). Because it involves a platelet disorder, ITP is known as a thrombocytopathy (Maton 1993) and because it involves a decrease in platelet function it is also known as a thrombasthaenia.

There were many cases in which low platelet numbers in patients with thrombocytopenic purpura had no identifiable cause, and this form of the disease became to be known as "idiopathic" – hence the full name idiopathic thrombocytopenic purpura, or ITP. However, Harrington and Hollingsworth established an immune pathogenesis for ITP when they gave healthy human volunteers serum from ITP patients (Harrington 1951) and described a circulating antiplatelet factor. Further *in vivo* studies clarified the involvement of IgG against platelets (Shulman 1965; Shulman 1965), and it became clear that the numbers of platelets drop because they are removed from the circulation. Because of this immune basis of the disease, idiopathic thrombocytopenic purpura is also known as immune-mediated thrombocytopenic purpura.

I–2 Incidence and Epidemiology of ITP

There are estimated to be 10 to 125 new cases of ITP per million of the population every year (George 1996). In the USA, annual rates for adults are approximately 66 cases per million and for children are approximately 50 cases per million. There are about 10 cases of chronic refractory ITP per million per year. Figures from outside the USA show approximately 10-40

cases in children per million per year in Denmark and the UK and approximately 125 cases per million per year in Kuwait (Silverman 2010).

I–2.1 Race

A genetic influence in the aetiology of ITP is likely, but further studies are required to verify the effect of race or ethnicity. There is some evidence that fewer African–American people are diagnosed with it (although this might be because the presence of petechiae and ecchymoses may not be noticed). A report by (Terrell 2005) added to evidence obtained since the 1960s that the proportion of black people with ITP was lower than the proportion of black people in the general population of the United States, and that the worldwide occurrence of ITP is rare among black Africans and people of African ancestry (Block 1966; Akwari 1987; Guthrie 1988; Salawu L 2001; George 2003; Aledort 2004).

I–2.2 Age

Age factors are relevant in the development of chronic and acute forms of ITP, which tend to affect different age groups. Around half of the 50–100 new cases seen per million people per year are in children, and this tends to be the acute form (platelet counts of less than 27,000 per µl), especially in children younger than 10 years old. The chronic form (platelet counts of over 27,000 per µl) is generally seen in older patients (Cines 2002). In a major study in 2000 children aged up to 5 years old from 38 countries, diagnosis of ITP in children with platelet counts of less than 20,000 per µl was shown to peak in the spring and early summer. There

was only a slight difference between boys (55%) and girls (45%) (Kuhne 2001). Age over 10 years is a risk factor for developing the chronic form of the disease (Watts 2004).

I–2.3 Gender

Childhood cases are roughly the same across genders (Kuhne 2001), but in adults, ITP tends to affect more women than men, in a ratio of 1 man to every 1.2–1.7 woman. In adults, with an annual incidence of 32 cases per million of the population, the average age of ITP diagnosis is 56–60 years (Neylon 2003). It is usually seen in its chronic form in adults, occurring particularly in middle-aged women (Cines 2002). Approximately 60,000 adults suffer from the chronic form of ITP in the USA alone (Amgen 2008).

I–2.4 Remission

Remission is seen in about 70% of children after 6 months (Ou 2006; Treutiger 2007) and some 30% of chronic cases undergo remission during follow-up. Another 30% are left with "mild" thrombocytopenia (Watts 2004). In adults, there is a high chance of remission in 20–40% of cases (Stevens 2006).

I–2.5 Mortality

The mortality rate of chronic ITP patients varies, but generally it increases with increasing age. In a study of ITP patients in the UK, mortality was approximately 60% higher than in people without ITP of the same sex and age, with an increased risk of death affecting 96% of patients aged 45 years or more (Schoonen 2009).

I–3 Definition and Types of Thrombocytopenia

Thrombocytopenia refers to low levels of platelets in the circulation, from any cause. A normal (healthy) count is somewhere between 150,000 and 450,000 platelets per μl of blood. A count of less than 10,000 is usually regarded as a medical emergency, and someone with 10,000–20,000 platelets per μl will generally receive some kind of treatment. Those with counts of 20,000–50,000 are evaluated on a case-by-case basis. However, counts above 50,000 are not usually treated (Cines 2005).

The accurate diagnosis of the thrombocytopenia in ITP requires exclusion of other kinds of thrombocytopenia. There are four types – alloimmune (or isoimmune) thrombocytopenia, drug-induced thrombocytopenia, secondary immune thrombocytopenia and autoimmune thrombocytopenia (Beutler 1995; Stiene-Martin 1998).

I–3.1 Alloimmune Thrombocytopenia

This can affect newborns (neonatal alloimmune thrombocytopenia) or any patient after a blood transfusion (post-transfusion purpura). In neonates there is an immune process involving an "antigen" (the region an antibody recognises and binds to, usually on a foreign pathogen) known as platelet antigen-1 (PLA1) – a polymorph of one of the glycoproteins found on a particular glycoprotein present on platelet membranes (GP IIIa) – so this is an immune process directed against a "self-antigen". Maternal antibodies against foetal platelets that express PLA1 are formed in mothers who are homozygous for a different platelet antigen, known as PLA2. These antibodies are then transferred through the placenta into the developing foetus to cause the disease in the newborn (Barron-Casella 1994). In the post-

infusion form, differences between the donor's and recipient's alloantigens induce the production of alloantibodies against platelets. These antibodies against the donor antigens are stimulated by the human platelet antigens (HPA).

I–3.2 Drug-induced Thrombocytopenia

Drug-induced thrombocytopenia occurs with drugs like quinine and heparin. Antibodies may be produced against the drug–platelet complex or the plasma protein–drug complex bound to platelets, and some drugs cross-react directly with platelets to cause myelosuppression (Beutler 1995; Stiene-Martin 1998).

I–3.3 Secondary Immune Thrombocytopenia

This is seen within the context of another primary disease, such as systemic lupus erythematosus, rheumatoid arthritis, leukaemia, von Willebrand factor deficiency, antiphospholipid syndrome, liver cirrhosis, HIV or hepatitis infections (Beutler 1995; Stiene-Martin 1998). Viral infections and other primary causes affect 5–10% of suspected cases (Bloom 1981; Cines 2005; Cines 2005).

I–3.4 Autoimmune Thrombocytopenia

Autoimmune thrombocytopenia is the fourth kind of thrombocytopenia, of which idiopathic thrombocytopenia is a common presentation. It is characterised by the destruction of antibody-coated platelets in the spleen or the liver, with a normal or slightly increased number of the megakaryocytes (platelet precursors) in the bone marrow (Semple 1998).

I–4 Chronic and Acute Forms of ITP

The disease can be acute and self-limited or chronic, depending on the duration of the thrombocytopenia. The acute form lasts for less than 6 months and usually occurs in children. The chronic form lasts for years and is usually found in adults (Bloom 1981; Beutler 1995; Stiene-Martin 1998). The risk of developing the chronic form can be predicted on the basis of platelet counts over 50,000 per μl and age over 10 years (Watts 2004). Immunoglobulins of the classes IgA, IgG and IgM are found in varying amounts in the chronic and acute forms of the disease.

The pathological mechanism of chronic ITP involves phagocytosis of platelets via autoantibodies after antigens on the platelet surface have become "antigenic". The first evidence of such autoantibodies was provided by Leuwen and colleagues (van Leeuwen 1982). Chronic ITP may also involve complement-mediated lysis and binding of autoantibodies to megakaryocytes, with affects on megakaryocyte colony formation and the production of platelets (thrombocytes) in the process called thrombopoiesis (McMillan 1978; Hoffman 1985).

I–5 Pathophysiology of ITP

While the significance of substances like serotonin and thrombopoietin have yet to be fully determined in the pathophysiology of ITP (Yanabu 1991), platelets are well known to be the major mechanism behind the development of thrombocytopenia.

Platelet destruction and abnormal platelet development and function bring about the characteristic purpura and coagulation disorders seen in ITP (Harrington 1951; Semple 1998), and it is the formation of antiplatelet antibodies that are key to this process (Van Leeuwen 1982). These antibodies against the body's own components, known as autoantibodies, are made specifically against various complexes of glycoprotein molecules in the membranes of platelets (Van Leeuwen 1982; Beardsley 1984; Woods 1984). The platelet-specific autoantibodies are destroyed by phagocytic cells.

Platelet-specific autoantibodies may lead to enhanced destruction of platelets, with the spleen, the liver and the bone marrow playing central roles in the pathology of thrombocytopenia. The spleen is the site of antibody production as well as platelet destruction, the liver is the site of destruction in severe cases of thrombocytopenia, and the bone marrow is the site of antibody production (Panzer 1986; Najean 1997).

Antiplatelet antibodies may have other effects. They may trigger platelet activation via various receptors on the platelet surface (Anderson 1991; Rubinstein 1991; Berndt 1993; Deckmyn 1998), and may interact with megakaryocytes in the bone marrow and thus impair production of more platelets (Ballem 1987; Hasegawa 1995; Nagasawa 1995).

Other factors may be involved in the peripheral platelet destruction seen in ITP. Platelets express class I molecules of the major histocompatibility complex (MHC), but some of the human leukocyte antigens (HLA) are increased in ITP patients (Helmerhorst 1982; Gratama 1984; Porges 1985; Mueller-Eckhardt 1989; Nomura 1998).

A genetic relationship has been suggested between specific platelet alloantigens and thrombocytopenia with an autoimmune basis (Song 1997). For example, genes for certain antigens of the HPA system (human platelet antigens) have been shown to have associations with autoimmune thrombocytopenia and ITP (Song 1997; Thude 1999).

Our understanding of the autoimmune nature of the destruction of peripheral platelets in ITP dates back to the 1950s (Harrington 1951; Shulman 1965), but to appreciate this complex phenomenon the functioning of the immune system needs to be explained fully.

I–6 Clinical Findings in ITP

ITP is an immune disorder with an autoimmune basis for the thrombocytopenia. Patients with ITP may be symptomless, or they may have skin symptoms, as well as a number of clinical and laboratory findings. Signs might include petechiae and purpura (skin rashes) usually on the hands or feet, epistaxis (nose bleeds), spontaneous bruising, gastrointestinal bleeding, genitourinary bleeding, heavy menstrual bleeding and, in more severe cases, intracranial bleeding. However, bleeding is not always seen in chronic ITP patients, even with low platelet counts (Olsson 2002). Certain signs that might be expected, like an enlarged spleen (splenomegaly), are rarely seen in ITP patients, even though the macrophages that destroy platelets are produced in the spleen.

Unfortunately, none of the signs described here are specific for the disease. The bleeding disorders usually occur when platelet counts drop to very low levels (less than 50,000) but

laboratory tests are required for making a proper diagnosis (Brighton 1996) as well as the exclusion of other forms of thrombocytopenic purpura

I–6.1 Platelet Count

There tends to be a lower peripheral platelet count in patients with chronic ITP (Beardsley 1989), and this thrombocytopenia occurs because of the shortened lifespan of platelets due to increased levels of destruction. However, platelet numbers are more helpful for monitoring the progress of ITP patients, rather than for diagnosis, because the diagnosis of ITP does not always depend on the absolute number of platelets.

I–6.2 Haematocrit

This is normal in ITP unless bleeding has occurred.

I–6.3 Peripheral Blood Smear

Microscopic examination of a blood film may reveal abnormal platelets. A greater proportion will be enlarged (over 2.5 μm) and immature (Bloom 1981). The volume and density of platelets are normally unrelated to their age, but after episodes of acute bleeding newly synthesised platelets are larger, with increased density and increased content of RNA (Ingram 1969; Ault 1995). Patients demonstrating high levels of peripheral platelet destruction have platelets with a greater mean volume (Martin 1983).

I–6.4 Bleeding Time

Bleeding time is a direct test of platelet function. It is often prolonged in ITP patients with minor lacerations or abrasions, but it can be normal in these patients. For this reason it should be not used for making the diagnosis of ITP, especially as there are many other diseases that cause prolonged bleeding time such as disseminated intravascular coagulation, Bernard–Soulier disease and Glanzmann's thrombasthenia (Davidson 2002).

I–6.5 Serotonin Levels

Platelets produce serotonin, store it within granules, and release it when the platelet is activated as part of the haemostatic response. There is some evidence to indicate a relationship between production of serotonin (5-hydroxytryptamine) and low platelet counts in children with ITP (Yanabu 1991). This association is not yet clear, but may have some relation to the inhibition of secretion of serotonin from platelet granules by alloantibodies (alloantibodies are antibodies against other people's antigens).

I–6.6 Bone Marrow Examination

Usually normal or slightly raised levels of megakaryocytes (the precursors of platelets) may be observed (Semple 1998). Megakaryocytes may be enlarged and immature with reduced evidence of platelet budding. In patients with a high level of peripheral platelet destruction, the megakaryocytic cytoplasm and nuclear DNA increase in quantity. The mean platelet volume in bone marrow blood is higher than in peripheral blood (Martin 1983).

I–6.7 Antiplatelet Antibodies

Some antiplatelet antibodies may be detected in the serum of ITP patients, showing increased levels of platelet-associated immunoglobulin (IgG). Assays such as the monoclonal antibody-specific immobilisation of platelet antigens (MAIPA) have a specificity of 80% and an accuracy of 85% (Wadenvik 1998; Winiarski 1998), however platelet antibody assays are generally considered unnecessary for diagnosis, although they are useful, and may be associated with disease severity. Any studies that characterise autoantibodies against platelet glycoproteins in ITP are likely to be useful for making an accurate clinical diagnosis .

I–6.8 Thrombopoietin Levels

Levels of the platelet growth factor thrombopoietin may have some value in ITP patients, especially as a marker for differentiating between thrombocytopenia from peripheral destruction and from failed thrombocytopoiesis. Levels are slightly raised in ITP patients (Kosugi 1996). They are low for any given platelet count, and a deficiency of the hormone may be involved in the pathophysiology of thrombocytopenia (Kosugi 1996; Kuwana 2001).

I–6.9 Reticulated Platelet (RP) Count

These young circulating platelets with a high nucleic acid content can be detected by flow cytometry. The number usually rises when the platelet count falls below 6–8 x 10^{10} per ml (Saxon 1998). (Shinjo 2005) found that the number of reticulated platelets detected by automated haematology analysis was significantly raised in patients with ITP. Reticulated platelet number allows estimation of megakaryocyte stimulation and platelet production

(Ingram 1969; Kienast and Schmitz 1990; Ault 1992), and may help discriminate between thrombocytopenia of different causes.

I–6.10 Mean Platelet Volume

The mean platelet volume (MPV) quantifies the average size of platelets found in blood. The average size of platelets is greater when they are being produced in higher numbers than normal, so the MPV represents platelet production in bone marrow. The MPV correlates strongly with increased platelet production. The mean platelet volume in blood from the bone marrow blood is higher than that in peripheral blood (Aliberti 1996). However, results can be conflicting. MPV can be very low in thrombocytopenia from impaired platelet production, but high in thrombocytopenia with peripheral destruction (as in ITP).

SECTION II: IMMUNE MECHANISMS

II–1 Immune System and Autoimmune Responses

The immune system is responsible for protecting the human body against pathogenic organisms, and can be considered in two parts: innate immune system, comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner, and the adaptive immune system, which is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges. The adaptive immune system is composed of the cell-mediated immune response and the humoral immune response (Alberts 2002). An effective immune response depends on the fine control and regulation of a complex series of events in both parts (Goldsby 2000). The humoral response involves production of antibodies in response to a challenge by an antigen, and it stimulates the cell-mediated response. The functioning of the humoral response is fundamental in the pathophysiology of ITP.

When the immune system reacts to the body's own antigens this is called autoimmunity. A "self-antigen" may be recognised by the host's immune cells as a foreign entity and autoimmune disease may develop. Genetic and environmental factors are involved in the aetiology of autoimmune diseases, such as the antigens of the major histocompatibility complex (MHC), defective T-cell function, and the breakdown of immune tolerance (Moller 1998), although a genetic basis for ITP has not been established.

Some autoimmune diseases involve antigens that are non-specific, in others they are organ-specific (Stefanova 2002; Saunders 2007) and in ITP they are specifically against components

of the glycoprotein complexes found in platelet membranes. In chronic ITP, an intravascular platelet antigen develops from a self-antigen or foreign molecule. Viruses may have a role in the aetiology of autoimmune thrombocytopenia, through some disturbance in the host's response, possibly involving platelet injury as a result of antigen-antibody complexes that originate from an immune event that is unrelated to the platelet (Kaplan 1992). This is known as the "innocent bystander" hypothesis.

The initial immune response occurs in the spleen, which is the prime site for dealing with intravascular antigens (Askonas 1958; McMillan 1974; Fujisawa 1993). Antigen-specific memory cells develop, leading to a response in which antiplatelet antibodies are created, first in the spleen, and later in the bone marrow. These antibodies will be of the immunoglobulin class known as IgG. They will bind to platelet-associated antigen to bring about its destruction and they will bind to antigens associated with megakaryocyte cells and thus may suppress future thrombopoiesis (production of platelets). A number of cell-mediated immune (CMI) reactions will also be stimulated, including the production of protein mediators known as cytokines. However, more evidence is needed about the defects in immunoregulation that are likely to contribute to the development of ITP (Semple 1995).

II–2 Cell-Mediated Responses

Cell-mediated immunity is the part of the immune response that does not involve antibodies or complement proteins. Instead, it involves the activation of macrophages, cytotoxic T cells (CD4 cells), and the release of various cytokines. There is some evidence that T-cell-

dependent cytotoxicity of target platelets occurs (Quagliata 1979), but more information is needed about the precise role of the CMI in ITP.

II–2.1 Function of T Cells

T cells provide protection against different pathogens. They are white blood cells, derived from the thymus gland, which form an essential part of cell-mediated response. They have special receptors on their cell surface called T-cell receptors (TCRs) and there are several T-cell subtypes, each with differing functions. These include T helper cells, cytotoxic cells, memory cells, regulatory cells (formerly suppressor cells) and gamma delta cells.

T helper cells help out in various immunologic processes. For example, they help B cells mature into plasma cells and assist in the activation of cytotoxic cells and macrophages. They are also known as CD4+ cells because they express a protein known a CD4 on their surface. T helper cells are activated when they react with processed antigens on the surface of antigen-presenting cells (APC).

The autoimmune process in thrombocytopenia generally starts after activation of CD4+ T helper cells, and the number of cytotoxic (CD8+) cells increases, which may cause an imbalance in the immune response. The formation of autoantibodies by B cells is then stimulated (Semple 1998). In the case of ITP, pathological antibodies are raised against platelets.

II–2.2 Cytokine Production

Activated T helper cells divide rapidly and differentiate further into more subtypes. The precise types of cell formed (Th0, Th1 or Th2) will depend on what signals they receive from the antigen-presenting macrophages or other APC cells. While the Th0 cells are precursors of Th1 and Th2 cells, the Th1 and Th2 cells will start to produce various cytokines. Cytokines are small proteins, peptides or glycoprotein molecules that regulate intercellular communication and act as cytokines as immune modulators. Examples of cytokines are the interleukins and interferons.

Th1 cells secrete interleukin IL-2, interferon gamma (IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNFα). Th2 cells produce a number of interleukins IL-4, IL-5, IL-6 and IL-10. Patients with ITP generally have higher levels of cytokines produced by Th1 and Th2 cells as well as cytokines from other sources. They include the IL-2, IL-4, IL-6, IL-10 and IL-11 as well as IF*N*-γ, migration inhibition factor (MIF), macrophage colony-stimulating factor (M-CSF) and GM-CSF (Joutsi-Korhonen 2000). Some of these cytokines induce Th1 and Th2 cells. MIF is involved in inhibition of lymphocytes and migration of macrophages (Clancy 1972). M-CSF induces phagocytosis and production of IL-1, and this may contribute to the higher rate of platelet destruction seen in ITP patients (Winiarski 1998).

Three cytokines with a major role in the immune response are platelet-activating factor (PAF), platelet-derived growth factor (PDGR) and transforming growth factor (TGF-β1).

These are chemoattractants for other immune cells like macrophages which are increased in ITP.

II–2.3 T cell Activation

The autoimmune process is triggered after activation of T helper cells. There are T-cell activators within the alpha-granules of platelets (Andersson 1998) so any damage to platelets might contribute to the overall immune response. Activators include the cytokine TGF-β1. This inhibits the activation and proliferation of both T and B cells. Any drop in serum levels of TGF-β1 in people with ITP may therefore lead to increased proliferation of both kinds of cells, so T-cell induction of B cells will lead to the production of more antibodies – in this case against platelets (Andersson 2002).

When Th0 and Th1 helper cells are activated, changes are observed among the different CD4+ helper cells. There is an *in vitro* reduction in $CD4^+$ suppressor–inducer (regulatory) cells, and an increase in $CD4^+$ helper-inducer cells. There are increases in CD8+ cytotoxic cells, which are seen as a change in the ratio of CD4+ to CD8+. There are also increased numbers of T cells activated by the DR subclass of human leukocyte antigens (the HLA system relates to the cell-surface antigen-presenting proteins of the major histocompatibility complex, or MHC, in humans). B cells are also induced to produce autoantibodies against platelets (Semple 1998).

The *in vitro* decrease in the T regulatory cell numbers may cause a diminished autologous mixed lymphocyte reaction (AMLR) in ITP. In fact, the AMLR was markedly decreased in 37 patients with chronic ITP compared to healthy controls in a study by (Deng 1993). The

percentage of DR+ T cells was significantly increased and correlated inversely with platelet counts and AMLR. This might signify some defect in immunoregulation in patients with ITP, and may relate to abnormal expression of surface DR+ antigen on non-T cells (Deng, 1993).

II–2.4 Antigen Presentation

T cells are activated by processed antigens on the surface of APCs (e.g.macrophages). There are three main types of professional antigen-presenting cell dendritic cells (DCs), macrophages and B-cells. Dendritic cells have the broadest range of antigen presentation, and are probably the most important APC. They are present in tissues in contact with the external environment, such as the skin (where there is a specialized dendritic cell type called langerhans cells) and the inner lining of the nose, lungs, stomach and intestines. They can also be found in an immature state in the blood. Once activated, they migrate to the lymph nodes where they interact with T-cells and B-cells to initiate and shape the adaptive immune response. Activated DCs are especially potent T_H cell activators because, as part of their composition, they express co-stimulatory molecules such as B7 (Banchereau 1998). They express both MHC I and MHC II.

Macrophages are white blood cells produced by the differentiation of monocytes in tissues. Macrophages function in both innate immunity as well as help initiate adaptive immunity of vertebrate animals. Their role is to phagocytose cellular debris and pathogens, either as stationary or as mobile cells. They also stimulate lymphocytes and other immune cells to respond to pathogens. After digesting a pathogen, a macrophage will present the antigen of

the pathogen to the corresponding Th cells. The presentation is done by integrating it into the cell membrane and displaying it attached to an MHC class II molecule.

B-cells, which express (as B cell receptor) and secrete a specific antibody, can internalize the antigen, which bind to its BCR and present it incorporated to MHC II antigens.

MHC antigens are also known as MHC moleculesand are coded for by genes in the major histocompatibility complex, which relates to the HLA system in humans, a gene-dense region of the genome that generates diversity into the immune repertoire. The MHC molecules were discovered historically as antigenic factors in organ transplantations, and the major MHC antigens are essential for immune function.

There are three classes of MHC molecules: class I, class II and class III, and each type consists of two polypeptide chains, an α and a β glycoprotein. MHC class I and II are expressed on the surface of different kinds of cells, including platelets.

Class I MHC molecules consist of a large glycoprotein α chain (encoded by class I MHC genes) and a small invariable protein called β2-microglobulin (encoded by a gene outside the MHC). The molecules are expressed on the surface membrane of nearly all nucleated cells and platelets. Platelets only normally express class I MHC molecules, however most studies on patients with ITP show no change in the frequency of HLA antigens corresponding to MHC class I (A, B and C) (McMillan 1980; Gaiger 1994). As there is great variety in the amino acid residues of the hypervariable regions of HLA molecules, any differences in HLA molecules may affect the development of autoimmune diseases (Janeway 1999).

Class I molecules present proteins to cytotoxic T cells by forming an MHC–protein complex with fragments of protein from any "self" or foreign protein synthesised within the cell, and displaying the complex on the cell or platelet surface. It can then be recognised by a T cell. If the cytotoxic cell recognises the displayed complex as "non-self" it will destroy the infected cell, and any other cells displaying the same protein. A "self–MHC" complex will be recognised by immunocompetent T cells and dendritic cells (Janeway 2001).

Class II molecules are heterodimers, consisting of two non-covalently associated glycoproteins, the α and β chains. They are encoded by different class II genes. They are expressed primarily on the surface membranes of macrophages (APCs) and B cells. They also present antigens, this time to T helper cells, inducing them to multiply and stimulate antibody-producing B cells to produce antibodies to the displayed antigen, and so stimulating an immune reaction from other cells.

Class III molecules are not like class I and II MHC molecules because they are not membrane proteins and they are not involved in antigen presentation (Wonsiewicz 1991). They are actually soluble protein products, all of which are all important in the immune process. Examples of class III molecules are the proteins of the complement system, tumour necrosis factor and the enzyme steroid 21-hydroxylase.

Platelets are associated with many immune-mediated diseases. Platelets from people with ITP present peptides from general cellular proteins and platelet-specific proteins to the immune system via MHC class I molecules. characterised several MHC class I-associated peptides on the platelets of patients with ITP. One of these peptides correlated with the B7 allele of the

HLA system, which relates to the platelet membrane glycoprotein GP Ib (Hopkins 2005). The HLA-associated molecules present the antigen fragments to the T cells, so any changes in the HLA system may have an effect on T cell immune function (Janeway 1999).

II–3 Humoral Responses

Antibodies are produced by specialist immune cells known as B cells, which are part of the humoral immune process (Pier 2004). The antibody response in ITP involves the production of autoantibodies against glycoprotein complexes on the surface of platelets (Semple 1998), and leads to disruption of the body's haemostatic mechanisms. The area the antibody recognises on a "foreign" particle is known as the antigen (Janeway 2001). In ITP this is a distinct region on one of the platelet's glycoprotein complexes. The specific part of the antigen that it binds to is called the epitope. When an antigen or self-antigen like a platelet glycoprotein is recognised or bound to by an antibody, it becomes "marked" for attack by a host of other immune mediators or the antibodies directly neutralise or deactivate it (Rhoades 2002; Market 2003).

It is possible that platelet injury occurs with antigen–antibody complexes formed during an immunological event that is essentially unrelated to the platelet (the "innocent bystander" hypothesis) (Kaplan 1992).

II–3.1 Function of B cells

Autoimmune processes like those involved in ITP generally start after activation of CD4+ T helper cells. T cells stimulate the formation of antibodies and autoantibodies by other

white blood cells known as B cells. These cells are produced and mature in the bone marrow in humans. The mature cells assist in the activation of cytotoxic cells and macrophages and the synthesise antibody (immunoglobulin), which is displayed on their cell membrane. When unbound antigen in the blood attaches to the membrane-bound antibody, the B cell is activated (Parker 1993; Verma 2007). The membrane-bound antibody forms part of the so-called B-cell receptor (BCR), which will be unique for a specific antigen and is a cell-signalling transducer (Wintrobe 2004). When the antigen binds to the membrane-bound antibody, it causes the BCRs to cluster together, lifting them away from other competing cell-signalling influences on the cell surface (Wintrobe 2004; Tolar 2008).

Once activated in this way, the B cells undergo several episodes of proliferation. At every stage, the BCRs may be subjected to point mutations, mostly of the substitution types (Oprea 1999), thus introducing diversity to the next generation of cells. Two kinds of cell are produced in large numbers. These are plasma cells and memory cells. The memory cells serve to "remember" the challenging antigen, which they do for many years so that the immune system can react quickly if the same antigen re-enters the body (Borghesi 2006). The plasma cells produce more antibody.

II–3.2 Antibody Production

The antibody produced by plasma cells usually has the same specificity as the immunoglobulin on the surface of the original activated B cell. There will be some variety

in the daughter cells because of "class switching" of immunoglobulin class on the antibody or because of random mutations (Oprea 1999).

Antibodies are produced in soluble and membrane-bound forms (Parham 2005). The soluble antibodies are secreted into the bloodstream and body cavities where they will identify and neutralise an enormous range of antigens. The membrane-bound antibodies (also known as surface antibodies, surface immunoglobulins, or membrane immunoglobulins) remain attached to the membrane of the B cells, ready to attach more antigen. A single cell may have 50,000–100,000 copies on its surface (Wintrobe 2004). The plasma cells live for only a few days, but during that time they secrete up to 2000 molecules of antibody per second, which means that an enormous quantity of highly specific antibody is generated.

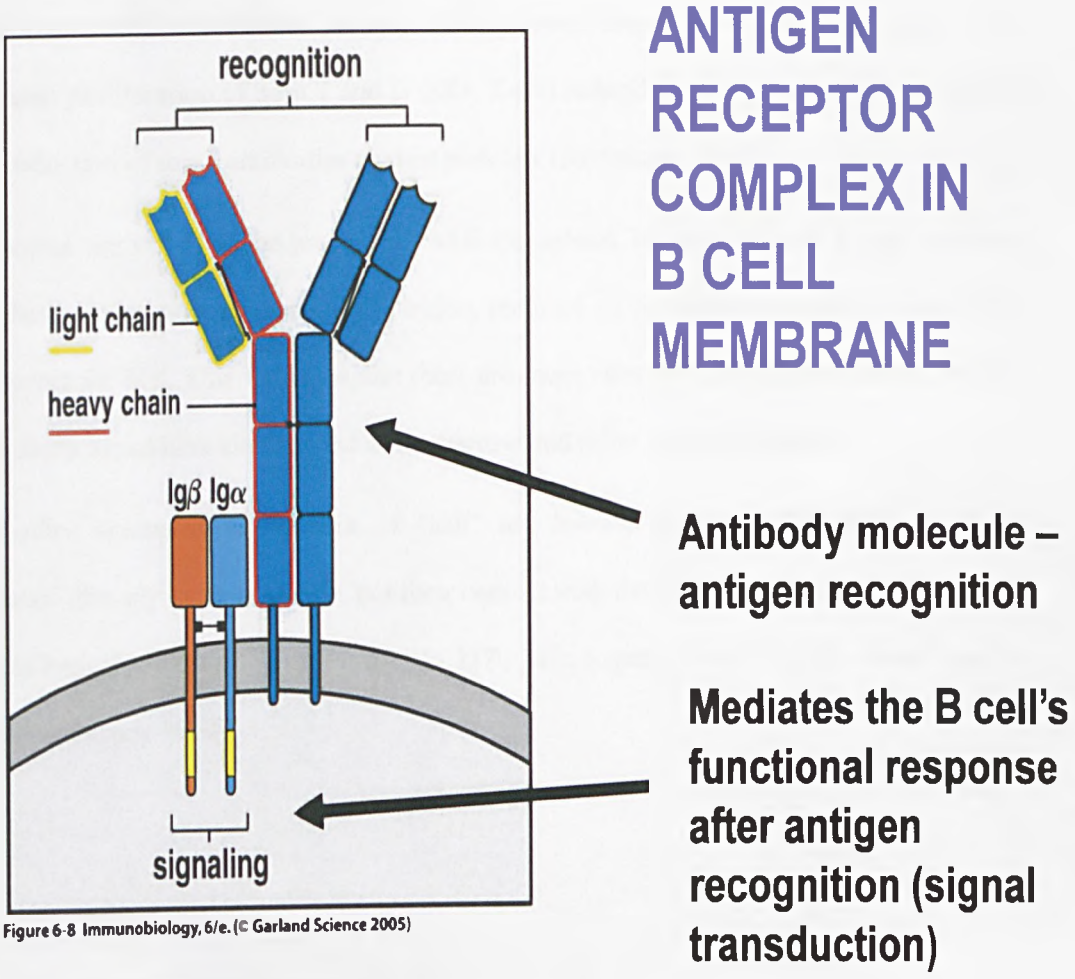


Figure III.1 Schematic of membrane antibody (antigen receptor) complex on B-cell

Antibody-producing B cells receive information via the macrophages and T cells (Roitt 1993) and from cytokines such as TGF-β1. This cytokine inhibits the activation and proliferation of T and B cells, so when serum levels drop in ITP patients, there may be increased proliferation of both T and B cells. T-cell induction of B cells will in turn lead to the production of more antibodies against platelets (Andersson 2002).

Antibodies are known to be produced within the spleen, but they are still found in people who have undergone splenectomy (surgical removal of the spleen), which is one of the treatments for ITP. This indicates that there are more sites of antibody production, and the most likely alternative sites are the bone marrow and other lymphoid tissues.

Antibodies against a component of "self" are known as "autoantibodies". They are produced like any other antibody, but their contact with their antigenic targets will result in a pathological condition in the body. In ITP, pathological antibodies are raised against platelets (Semple 1998).

II–3.3 Antibody Structure and Diversity

II–3.3.1 General Structure

Antibodies (immunoglobulins) are heavy, globular, Y-shaped plasma glycoproteins with a characteristic sandwich-shaped fold, and a mass of approximately 150 kDa (Litman 1993). They are made of sugar chains and amino acid residues, comprising 82–96% polypeptide and 4–18% carbohydrate (Mattu 1998).

Each antibody contains four polypeptide chains – two identical *heavy* chains and two identical *light* chains, connected together by disulfide bonds (Woof 2004). On the arms of the "Y" are two distinct regions known as the Fab and Fc regions. The Fc region, which stands for "fragment crystallisable", is on the base of the Y and the Fab region, which stands for "fragment antigen binding", is on the arm of the Y. On the amino-terminal end of the antibody there is a variable region known as the *paratope* which binds to a specific site on antigen and forms part of an "antigen-binding site". Other common structures of antibodies include the complementarity determining regions (CDRs) and hypervariable regions.

All antibodies share the same general structure (Woof 2004), but the immune system needs to respond to the millions of different antigens found on an enormous range of bacteria, viruses and fungi. Diversity among antibodies is essential for ensuring that an antibody exists for any unique antigen on a target pathogen . This structural diversity also means that antibodies are produced that are capable of binding to a specific part of a glycoprotein complex on the surfaces of a platelet, as in ITP, and generate an autoimmune response.

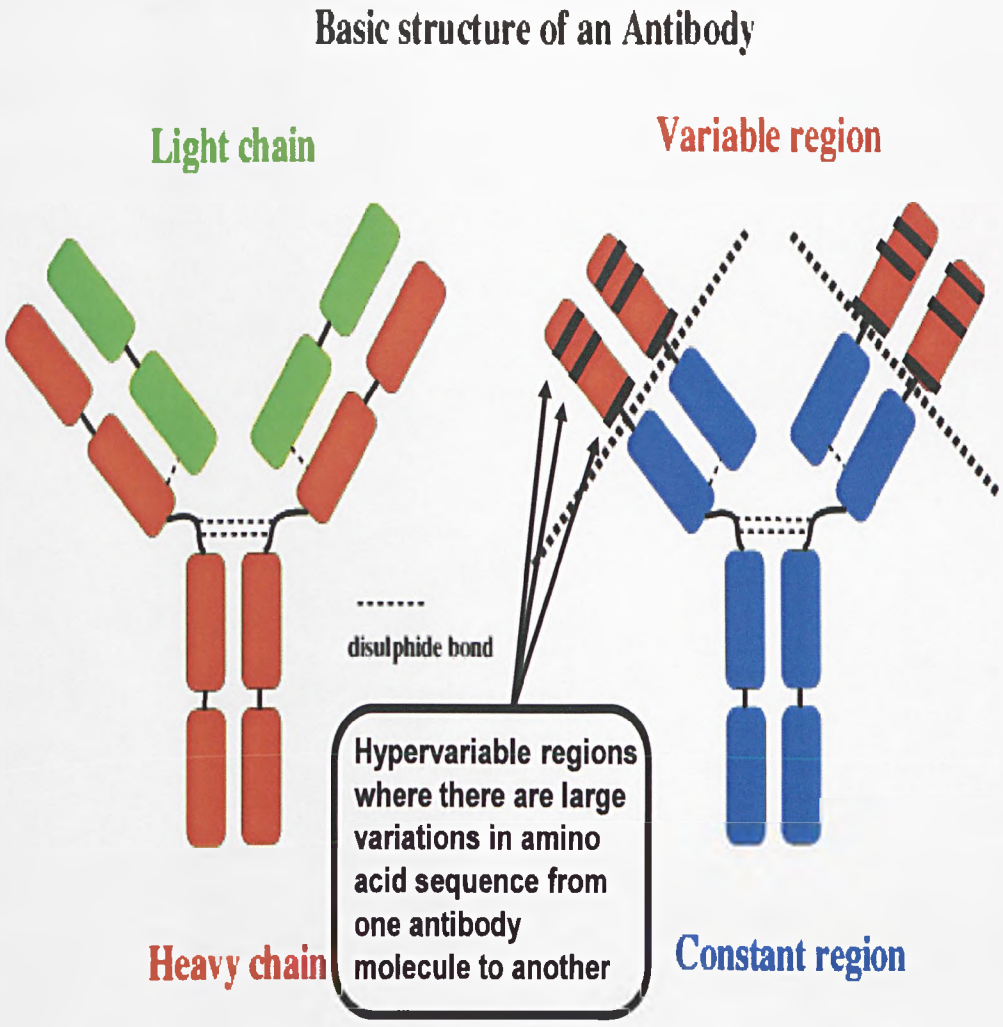


Figure III.2 Schematic of basic structure of an monoclonal antibody

II–3.3.2 Structural Diversity

Structural diversity among antibodies is achieved in various ways, primarily involving the hypervariable region and thus the antigen-binding sites, some of which are through spontaneous genetic means.

II–3.3.3 Variable Regions

Antibodies have a small, extremely variable region of 100–110 amino acids at the tip of their amino terminal. This region is known as the hypervariable (or FV) region. The part of the hypervariable region on the amino-terminal end of the antibody that binds to a specific site on an antigen is called the paratope. The differences in the hypervariable region relate to the loops in four polypeptide chains (known as heavy chains and light chains), with structural domains containing 70–110 amino acids (Barclay 2003). The hypervariable region is the most important region for binding to antigens. Small differences here produce millions of different antibodies. Antibodies containing only one kind of variable region are produced by B cells, so all the antibodies secreted from one cell will have the same variable region (Janeway 2001; Bergman 2004), but different B cells will produce antibodies with different variable regions. Diversity in the variable regions is governed by genetic mechanisms.

II–3.3.4 Heavy and Light Chains

There are two kinds of chain in the antibody – light and heavy – connected by covalent and non-covalent bonds. There are five types of heavy chain, which are known as alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ). These names correspond with the class of immunoglobulin (Ig) they produce, namely IgA, IgD, IgE, IgG and IgM (Janeway 2001). They have molecular weights of 50 kD or more and they vary in their amino acid content (450 to 550 each), size and composition (Janeway 2001). Heavy chains have two distinct regions – a constant (C) region and a variable (V) region. The variable region is about 110 amino acids long, with just one immunoglobulin domain. In IgM and IgE the constant region contains four immunoglobulin domains, and in IgG, IgA and IgD there are three immunoglobulin domains in a line together with a hinged region that gives the molecule flexibility (Woof 2004).

There are two types of light chain, and these are known as kappa (κ) and lambda (λ). They have molecular weights of about 25 kD, and consist of 211–217 amino acids (Janeway 2001). They have one constant and one variable domain.

II–3.3.5 Isotypes

The type of *heavy* chain determines the antibody's "isotype". Thus the alpha (α) chain is IgA, the delta (δ) chain is IgD, the epsilon (ε) chain is IgE, the gamma (γ) chain is IgM and the mu (μ) chain is IgM (Janeway 2001). The different isotypes have different functions and roles in different parts of the immune system. B cells at different stages of development will produce antibodies with different isotypes (Goding 1978; Market 2003). Antibodies are also subject to "class-switching" in which the heavy chain changes in

daughter B cells, producing antibodies with the same paratope but different immunoglobulin isotypes.

II–3.3.6 Idiotypes

The "idiotype" of an antibody is determined by the complementarity determining regions (CDRs). These form part of the antibody that structurally "complements" a specific antigen, so that they fit together like a lock and key, and facilitate antigen recognition by immunoglobulin (Li 2004). Three CDRs can be found on the variable (V), diverse (D), joining (J) and constant (C) domains of an antibody. Two of them, CDR1 and CDR2, are found in the V domain. The third, CDR3, is found on some V domains and some C domains and all of the D and J domains. D domains are found on the heavy chains only.

If viruses are involved in the development of autoimmune thrombocytopenia, as has been speculated, then it might be through the production of anti-idiotype antibodies (Kaplan 1992). There may be a defect in the T-cell induction of antibodies (Moller 1998) because of T cell responses to viral peptides during an infection There may be cross-reactivity between structurally similar parts of the viral antigens and platelet glycoproteins in the host (Moller 1998). There may be a disturbance in the host immune response relating to molecular mimicry, production of anti-idiotype antibodies, enhanced expression of the MHC molecules and changes in endogenous antigen (Kaplan 1992). Platelet injury may also result from antigen-antibody complexes that arise from an immune event that is not related to the platelet (the "innocent bystander" hypothesis).

II–3.3.7 Fab Region

The Fab region is part of antibody that has direct contact with antigen through the paratope on the tip of antibody, so is important in antigen binding. Antibody binding to platelets, and to their glycoprotein membrane complexes, can be Fab-mediated (Stricker 1986). The Fab region is composed of one constant and one variable domain from each heavy and light chain of the antibody (Putnam 1979) and each Ab has two binding sites for contacting with antigen. This means it has enormous specificity for antigens. Fab fragments are easy to isolate and produce experimentally, and are useful for characterising platelet glycoproteins.

II–3.3.8 Fc Region and Receptors

The Fc region is composed of two heavy chains with two or three constant domains (Huber 1980; Janeway 2001). Once binding has occurred, the Fc region recognises opsonised particles, causes cell lysis, and induces degranulation of mast cells, basophils and eosinophils (Heyman 1996; Woof 2004).

The Fc region binds to specialised protein receptors on an antigen known as Fc receptors. There are several kinds of Fc receptors (alpha, gamma and epsilon) that differ in their antibody affinities due to different molecular structures. The gamma subtypes are FcγRI, FcγRII and FcγRIII . The first of these, FcyRI, has the highest affinity for IgG, binding more strongly than FcγRII or FcγRIII. It contains an additional immunoglobulin-like domain so it can be activated by a single molecule of IgG (the other two need to bind more molecules of IgG to become activated) (Fridman 1991). The Fcγ receptors are necessary for phagocyte activation, the release of inflammatory mediators, and mediating the interactions of

monocytes, phagocytes, macrophages and other cells – including platelets – to bring about the ingestion, phagocytosis and destruction of IgG-opsonised (coated) particles (Indik 1995)).

On macrophages the Fc receptors mediate the recognition and binding of IgG-coated cells in the mononuclear-phagocyte system. This is important in ITP (Karpatkin 1992) because platelets coated in IgG will be quickly recognised by Fc receptors on macrophages. The Fc portion will fix to the tail of the IgG antibodies and the platelet will be phagocytosed in the liver or spleen (McMillan 2000).

II–3.4 Genetic Variability

Antibodies have to recognise epitopes on millions of different antigens in order to generate a suitable immune response, and there are a number of ways in which the immune system maintains this adaptability and diversity, primarily through genetic means. Much of the diversity among antibodies is introduced via the variable region, affecting their antigen-binding specificity. Random generation of these specificities through genes may contribute to the generation of autoantibodies seen in ITP.

The individual domains of each antibody are encoded on specific loci of specific chromosomes. In humans, the locus for genes of the heavy chain, for example, is found on chromosome 14. For the light chains, they are found on two separate loci on chromosomes 2 and 22. Individual paratopes are encoded by different gene segments, which are subject to random combinations together with random mutations (Diaz 2002; Market 2003).

Mutations occur during rapid proliferation of B cells, producing thousands of daughter cells with slightly different specificities. The cells with the greatest specificity for a particular antigen will then be selected to differentiate into antibody-producing plasma cells and memory cells.

Four important ways to maintain diversity in the antibody pool are class switching, somatic recombination, junctional diversity and somatic hypermutation, as described below. The combination of mutations and gene-based variations in antibody domains introduces immense variability (Widmann, 1998). However, hypermutation processes may also be involved in a cell "auto-selection" process that leads to the development of an autoimmune response.

II–3.4.1 Somatic Recombination

The CDRs in the variable regions of heavy chains are encoded by 65 genes. In somatic recombination, these genes combine randomly with the genes for other domains on the antibody (Parham 2005). The antibody produced by these developing B cells in the bone marrow will have a unique and complete variable region in each immunoglobulin chain, adding to the diversity in antigen recognition sites (Li 2004).

The process is also known as V(D)J recombination, because it relates to the regions encoded in different "gene segments", namely the variable (V), diversity (D) and joining (J) segments (Nemazee 2006). The D gene segments are short sequences of DNA that join the V and J gene segments. There is random selection of one V, D and J segment in each heavy chain (VH, DH and JH) and one V and J segment in each light chain (VL and JL).

Recombination occurs in several stages, so for the heavy chains the DH merges with the JH segment to form a DJH segment before combining with the VH segment.

There are multiple copies of each type of gene segment in humans, so the different combinations of segments will produce antibodies with different variable regions, with different paratopes and different antigen specificities (Market 2003).

II–3.4.2 Junctional Diversity

Junctional diversity occurs during somatic recombination, when gene segments fail to join correctly, and introduce variations into the DNA sequence. When the gene segments for the variable region are re-arranged, any unused segments are removed, which causes breakages between the segments. The broken ends form hairpin loops and join back together, but not precisely, resulting in the random addition or subtraction of nucleotides (Janeway 2001). Two recombination activating genes are required, together with various DNA repair proteins and enzymes (Wyman 2006).

II–3.4.3 Somatic Hypermutation

These are "point mutations" within the variable regions of the genes for both heavy and light chains. They are not completely random (Charles 2005). They tend to occur in the hypervariable regions of DNA that correspond to the CDR sites, which govern antigen recognition and thus create diversity in the recognition and binding properties of B cells (Janeway 2001; Li 2004). B cell receptors undergo these kinds of mutations (mostly substitutions) at a very high rate in response to signals from activated T cells (Oprea 1999).

Hypermutation is complex, involves a number of enzymes at different stages (Bachl 2006; Teng 2007), and can result in the production of non-productive genes.

II–3.4.4 Class Switching

The genes for antibodies may be re-organised by "class switching". In this process, an activated B cell will have one type of heavy chain but its daughter cells will have a different base in their heavy chain. In this way, antibodies will be produced with the same paratope but a different isotype, thus having a different function and role from the parent B cell.

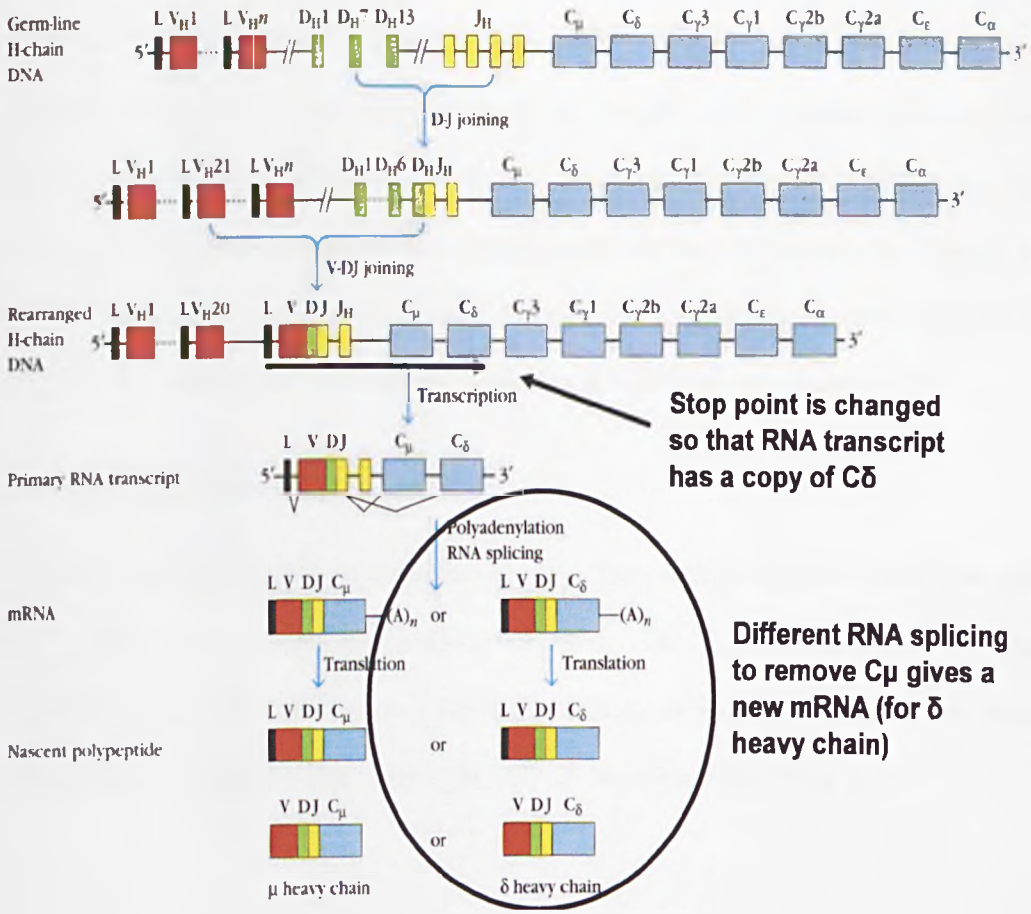


Figure III.3 Schematic of CLASS SWITCHING

SECTION III: PLATELETS

The role of platelets in the pathogenesis of ITP is complex. When a blood vessel wall is damaged, there are interactions between the vessel wall, plasma proteins, and the glycoproteins in the platelet membrane that arrest haemorrhaging and limit the vascular damage. Autoantibodies against the glycoproteins in platelet membranes disrupt a large number of processes involved in immune function and haemostasis, and are implicated in the aetiology of thrombocytopenia with an autoimmune basis such as occurs in ITP.

III–1 General Structure of Platelets

Platelets are small, regularly-shaped discoid cells. They measure just 2–3 μm in diameter and have a volume of 5–7 femtolitres (Majerus 1994). They do not have a DNA-containing nucleus. Their internal structure is complex, consisting of four zones, namely the peripheral zone, the sol–gel zone, the organelle zone, and the membrane zone (Fig. III.1).

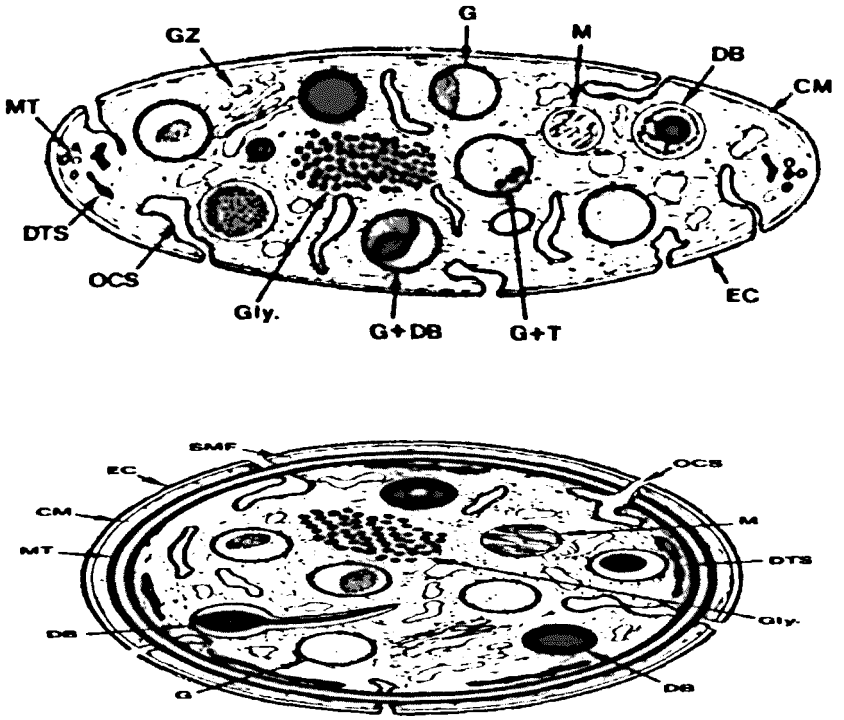


Figure III.4 ultrastructural features of a thin section of the platelet are illustrated. The peripheral zone includes the exterior coat (EC), trilaminar unit membrane (CM), and submembrane area containing specialized filaments (SMF). The platelet matrix is the sol-gel zone which is under the peripheral zone and contains act in microfilaments, structural filaments, the circumferential band of microtubules (MT), and glycogen (Gly). The elements of this zone include mitochondria (M), granules (G), and dense bodies (DB). . The membrane systems contain the surface-connected canalicular system (CS) and the dense tubular system (DTS)(White 1994).

The peripheral zone consists of the external membranes and the open canalicular system (OCS). The OCS is series of fine tubes that extend throughout the zone and connect to the outer surface, allowing communication between in both directions between the platelet and its environment.

The sol–gel zone is the contractile cytoskeleton of the platelet. It allows activated platelets to change from a discoid shape to a stellate shape, with surface projections known as pseudopods (Hoffbrand 2001), and causes internal contraction of the cell that cause the platelet granules to release their contents. The cytoplasmic domains of the glycoprotein complexes are connected to the cytoskeleton.

The organelle zone contains the metabolic components of the platelet. Among these are various granules that contain 30–50% of all the platelet's proteins, and secrete them on activation. Dense bodies consist of delta granules that store the potent platelet activator adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and contain calcium and serotonin taken up from the cytoplasm. Alpha granules contain adhesion molecules like platelet factor 4, TGF-β1, platelet-derived growth factor, fibronectin, beta-thromboglobulin, vWF, fibrinogen and coagulation factors V and XIII. Lambda granules contain hydrolytic enzymes. Platelet peroxisomes are involved the production of prostaglandins, and the mitochondria (usually seven in each platelet) are important in lipid metabolism (Beutler 1995).

The membrane zone is the outer plasma membrane layer. It is 7.5 nm thick, with a bilaminar structure made up of proteins, lipids (of which 75% are phospholipids) and carbohydrates.

The membrane bi-layer contains several complexes of glycoproteins (a type of integrin) and phospholipids, as well as substances that are important for platelet function, such as calcium and an enzyme for producing prostaglandins (White 1994).

III–2 Platelet Membrane Glycoproteins Structure and Function

There are at least nine different glycoproteins embedded in the platelet membrane. Among them are GP Ia, GP Ib, GP Ic, GP IIa, GP IIb, GP IIIa, GP IV, GP V and GP IX. They are embedded as complexes. They are all essential for maintaining haemostasis and normal immune responses(Beutler 1995; Stiene-Martin 1998). They all act as antigens to autoantibodies, but the major ones are GP Ia, GP Ib, GP IIa, GP IIb, GP IIIa and GP IIIb. In ITP these glycoprotein complexes become the self-antigens, and the generation of antiplatelet antibodies to the molecules is fundamental to the development of thrombocytopenia in ITP.

Some of the membrane glycoproteins are types of integrins. They are embedded as complexes within the phospholipid bi-layer of the platelet membrane and connected to the platelet cytoskeleton by their cytoplasmic domains. They have a heterodimeric structure, comprising a protein molecule attached to an oligosaccharide (carbohydrate) molecule, and two distinct chains known as the α (alpha) and β (beta) subunits. The carbohydrate molecule influences the folding of the protein and adds to its stability, and the chains form the ligand-binding site.

They interact with many substances such as collagen, fibronectin, vitronectin, thrombin, vWF and collagen, and facilitate adhesion of platelets with the damaged blood vessel wall as well as other platelets. They all have essential roles in the immune response in addition to tissue repair and homeostasis. Any disruption to their normal function is associated with dysfunctional platelet activity and contributes to the development of thrombocytopenia as seen in ITP.

The glycoprotein complexes form a range of specific receptors, thus:

- GP IIb/IIIa complex forms the fibrinogen receptor
- GP αV/GP IIIa complex forms the vitronectin receptor
- GP Ic/IIa complex forms the fibronectin receptor
- GP Ib–IX–V complex forms the vWF receptor
- GP Ia/IIa interacts with collagen.

III–2.1 GP IIb/IIIa Complex

Structure

The GP IIb/IIIa complex is a heterodimeric complex. The GP IIb subunit is an α-integrin and the GP IIIa subunit is a β-integrin (Honda 1998), with molecular weights of 145 kD and 90 kD, respectively (Beutler 1995). The complex provides about 18% of the total proteins of the platelet plasma membrane. The GP IIb (α) subunit is a transmembrane protein containing four

domains and a total of 1008 amino acids (Beutler 1995; Berndt 2000). The GP IIIa (β) subunit contains a carboxy-terminal domain, a transmembrane segment, a cysteine-rich *N*-terminal domain, and a total of 762 amino acids. The complex carries HPA-3, HPA-9 and Va.

The subunits are produced separately, then combined in a calcium-dependent process to form the complex within the rough endoplasmic reticulum (Tomiyama 1993; Beutler 1995)). The complex then binds to the transmembrane domain of the platelet via non-covalent calcium-dependent bonds (Page 1991; Berndt 2000).

Figure III.2 Schematic of the Glycoprotein IIb/IIIa Complex

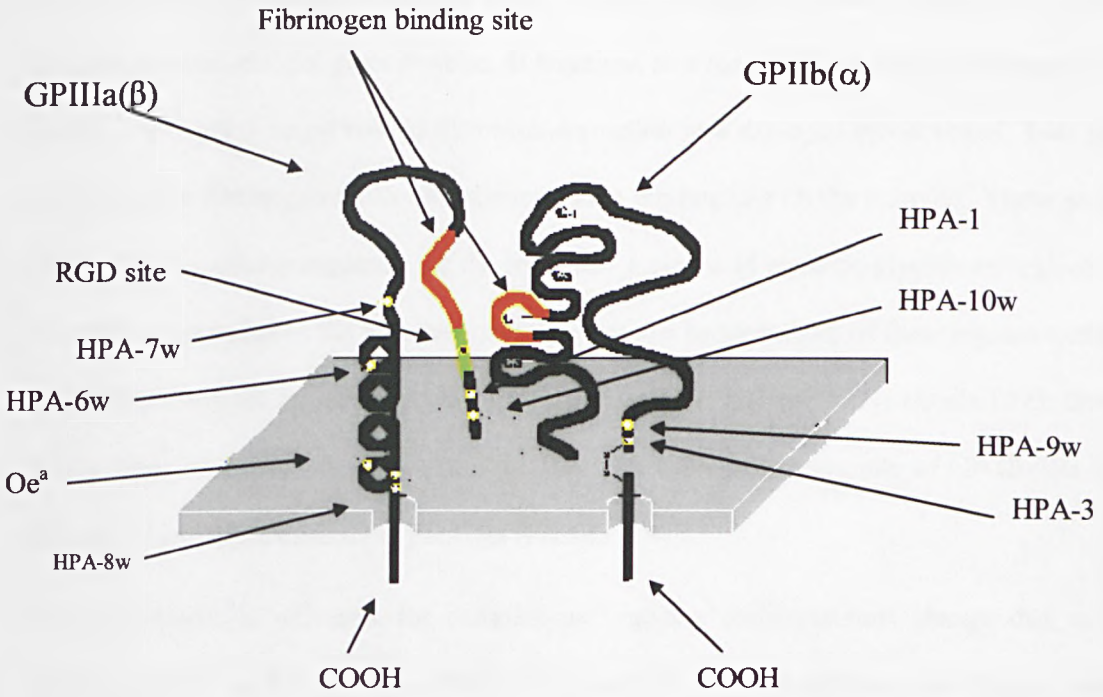


Figure III.5 Schematic diagram of the GP IIb/IIIa heterodimer complex. The fibrinogen binding site is located on both subunits of the glycoproteins. The binding site for the tripeptide RGD sequence (arginine-glycine-aspargine) is on GP IIIa. GP IIIa also contains the human platelet antigens HPA-1, HPA-4, HPA-6, HPA-7, HPA-8, HPA-10, –Gro[a] and –Oe[a]. HPA-3 and HPA-9w are located on GP IIbα (National Institute for Biological Standards and Control, 2002a).

Function

The GP IIb/IIIa complex is involved in the immune response and haemostasis and is one of the most studied platelet glycoproteins. It functions as a receptor for soluble fibrinogen in the plasma, so it has a major role in thrombus formation in a damaged blood vessel. Two main regions in the fibrinogen molecule interact with a binding site on the complex. These are the RGD (the recognition sequence for the tripeptide amino acid arginine-glycine-aspargine) and the carboxy-terminal of the gamma chain. Interactions between both of these regions with the Gp IIb/IIIa complex are necessary for the thrombus to form (Page 1991; Honda 1995; Berndt 2000). This interaction is shown in Fig. III.2. The calcium-binding site of GP IIb/IIIa may also act as a calcium channel in platelets (Rosado 2001).

When a platelet is activated, the complex undergoes a conformational change that makes fibrinogen bind to it with high affinity (Wagner 1996). The conformational change causes GP IIb-IIIa to be displayed differently on activated platelets. This interaction between the receptor and fibrinogen initiates the formation of a haemostatic plug, or clot. More platelets are then induced to aggregate in the subendothelium of the damaged blood vessel where they bind with vWF, vitronectin and fibronectin. However, if a compound containing or mimicking an RGD sequence links to the complex, it enhances the display of the complex on resting platelets or masks the thrombin-induced increased display of the complex (Marder 1993). Either way, it inhibits platelet activation. However the reaction can be reversed by adding more fibrinogen (Page 1991).

The binding of fibrinogen to activated platelet also induces the expression of new epitopes on the GP IIb/IIIa complex. These are called ligand-induced binding sites, or LIBS (Shattil 1985) and they are involved in transmembrane signalling (Honda 1995). Three distinct LIBS have been identified. Two are on the GP IIb subunit (one on the carboxy-terminus of the heavy chain and one on the *N*-terminus) and one is on the GP IIIa subunit. The ligand-binding activity of the GP IIb/IIIa complex depends on the change in the GP IIb/IIIa complex from an inactive state to an active state. This is induced by ADP and antiplatelet antibodies. As well as the conformational changes of the complex, the membrane undergoes certain modifications (Shattil 1985; Berndt 2000). Then the complex is able to transfer information from the inside of the platelet to the outside (Tohyama 1998), and this leads to a series of biochemical reactions, such as phosphorylation of cytoplasmic proteins in the cytoplasmic domain of the GP IIIa subunit by protein kinase C. Another conformational change follows in which the platelet cytoskeleton is rearranged, releasing activation factors from the alpha granules and dense bodies so that secondary aggregation occurs (Berndt 2000; Rosado 2001). As a consequence, more molecules of GP IIb/IIIa are exposed on the platelet membrane, and more fibrinogen binds to it (Hillery 1991; Law 1996). This situation involves both inside–outside and outside–inside signalling, and links ADP with activation of the GP IIb/IIIa integrin. The LIBS, fibrinogen and RGD all contribute to the formation of fibrin clots (Frelinger 1990).

Role in ITP

The GP IIb/IIIa complex is highly immunogenic, with several autoepitopes and alloantigens, and clinically important polymorphisms (Beardsley 1984; Woods 1984;

Woods 1984; Tomiyama 1987; Tomiyama 1989; Kunicki 1992; Kokawa 1993). There is evidence that the IIb component is involved in the aetiology of autoimmune thrombocytopenia (Stiene-Martin 1998; Kahn 1999; Moran 2000). Autoantibodies against the self-antigens in this complex are common in the sera of ITP patients (Van Leeuwen 1982; Beardsley 1984; Woods 1984).

III–2.2 GP Ia/IIa Complex

Structure

The structure of the GP Ia/IIa complex is shown in Fig. III.3. It has two subunits, the Ia subunit (α2) and the IIa subunit (β1), joined by non-covalent bonds. Both subunits consist of a single polypeptide chain with intra-chain disulfide bonds. The GP Ia subunit contains a transmembrane domain plus a short cytoplasmic segment and two human platelet antigens (HPA-5 and Sit^a). There is a collagen-binding site on the *N* terminus. (National Institute for Biological Standards and Control, 2002).

Figure III.6 Schematic of the Glycoprotein Ia/IIa Complex

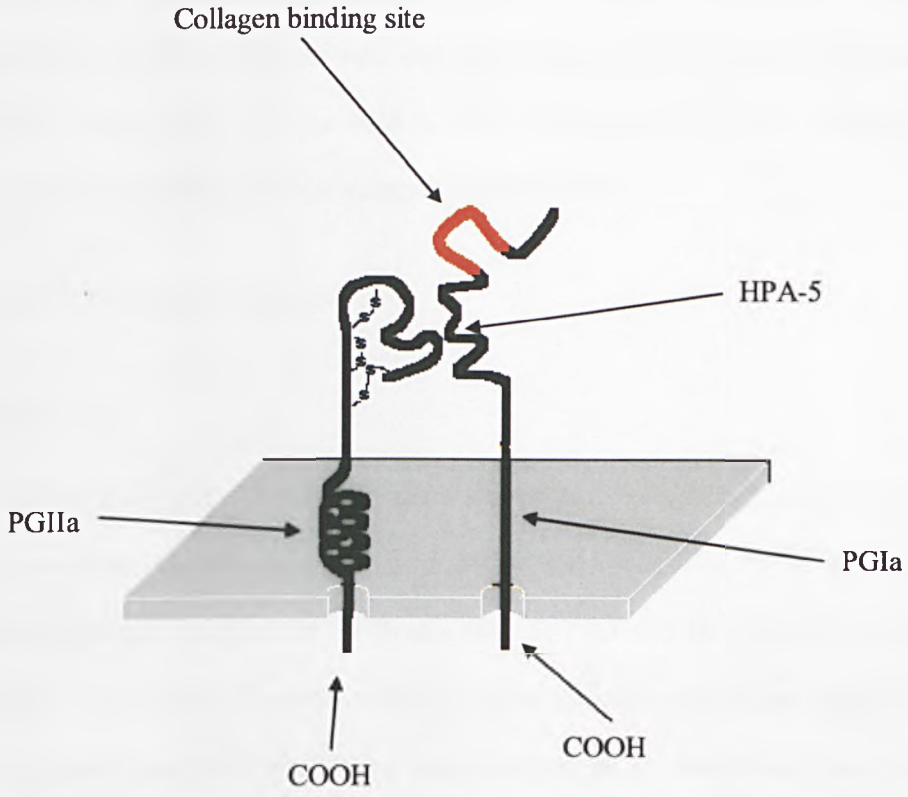


Figure III.6 The divalent, cation-dependent GP Ia/IIa complex showing the location of the human platelet antigens (HPA-5 and Sit[a]) and the collagen binding site (National Institute for Biological Standards and Control, 2002b).

Function

The GP Ia/IIa complex is an adhesion receptor and it acts as a collagen receptor. It has an important role in clot formation, promoting the adherence of platelets to the subendothelium of the blood vessel. Through its interactions with collagen, it tethers the platelet to the wall of the blood vessel wall and prevents them from being detached by the high shear stress exerted by the flowing blood. In conditions of high shear stress in the blood vessel, GP Ia adheres to vWF and the blood vessel subendothelium(Moroi 1997).

III–2.3 GP Ib/IX/V Complex

Structure

This hetero-oligomeric protein complex is produced from four genes. In this complex, GP Ib is non-covalently associated with both GP IX and GP V. The GP IX and GP V components are non-covalently linked to GP Ib in a ratio of 2:2:1 (GP Ib to GP IX to GP V). The GP Ib subunit is the largest fragment, with 610 amino acids and a molecular weight of 170 kD. It is composed of one α subunit (with a molecular weight of 144 kD) and one β subunits (with a molecular weight of 26 kD), linked by disulfide bonds. Both subunits are integral to the platelet membrane and contain cytoplasmic components (Macintyre 1987; Feng 2000; Moran 2000). Fig. III.4 shows the structure of the complex and the location of the alloantigens, HPA-2 on GP Ibα, and Ly^a on GP Ibβ. GP Ibα is related to actin-binding proteins in the sub-membrane of the platelet. The vWF and thrombin binding sites are on the N terminus (National Institute for Biological Standards and Control, 2002c).

Figure III.7 Schematic of the glycoprotein Ib/IX/V complex

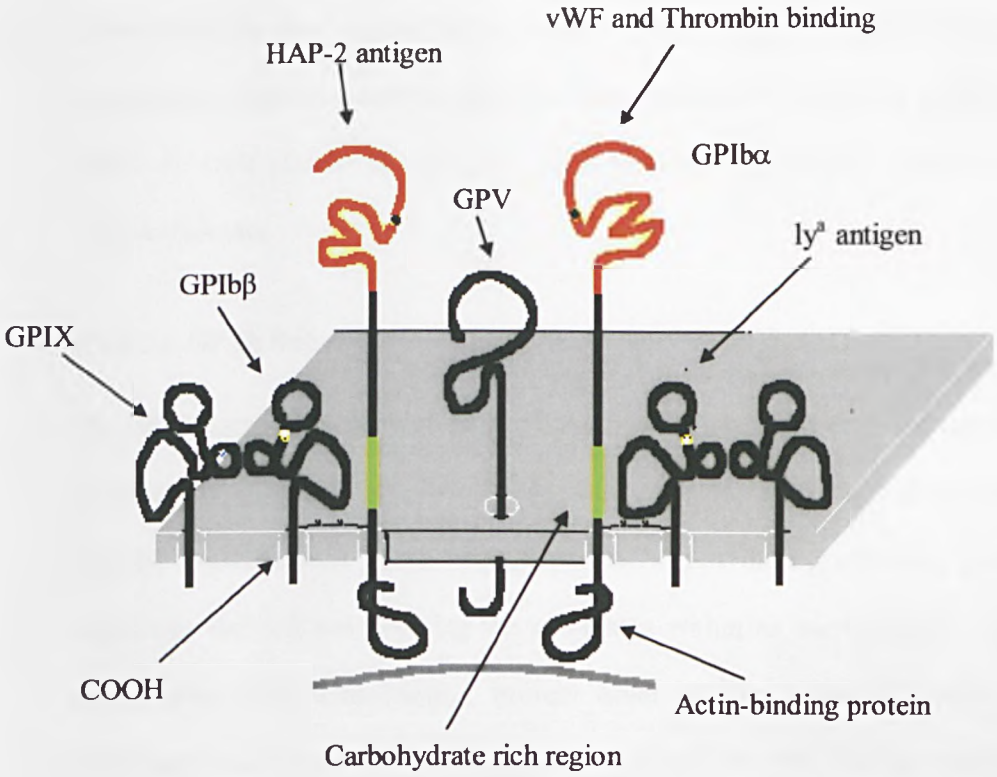


Figure III.7 Schematic diagram of the non-covalently associated GP Ib–IX–V complex, showing the locations of the human platelet antigen HPA-2 on GP Ibα, and the Ly^a antigen on GP Ibβ, and the vWF- and thrombin-binding domain sites on the N terminus. GP Ibα is also related to actin-binding proteins in the sub-membrane of the platelet (National Institute for Biological Standards and Control, 2002).

Function

The GP Ib/IX/V complex forms the receptor for vWF, therefore it is involved in interactions with vWF, as well as thrombin and actin, and facilitates platelet adhesion to vascular subendothelium after vascular injury. It initiates signalling events within the platelet that lead to enhanced platelet activation and thrombus formation. There are approximately 11,000 copies on each platelet (Kahn 1999). It is also expressed on the precursors of platelets – megakaryocytes.

III–2.3.1 GP Ib Subunit

The GP Ib component is involved in platelet interactions with vWF and thrombin through its extracellular domain. It also has binding sites for actin in its short cytoplasmic region. The globular protein, actin, forms microfilaments within the cytoskeleton, giving mechanical support to the cell and securing the cytoplasm within its surroundings to facilitate signal transduction. The actin-binding protein binds to the carboxy-terminal of the GP Ib component, and accelerates the aggregation response to vWF. During platelet activation the binding protein is degraded by endogenous calcium-activated proteins. Then GP Ib can move freely and facilitate the adhesion of platelets to immobilised vWF and the blood vessel subendothelium.

The adhesion of unstimulated platelets to vWF in the subendothelium of the damaged vessel wall (George 1984) occurs after a conformational change in the GP Ib that is brought about by high shear stress exerted by blood as it passes the tethered Gp Ib/IX component. Normally, platelets only interact with vWF bound to a solid surface under conditions of high shear rates

(found in arteries and arterioles) (Weiss 1978). This is a shear-dependent response and it may be regulated by the cytoplasmic tail of GP Ib (Macintyre 1987). When the GP Ib component is blocked with monoclonal antibodies, platelets do not adhere well to the subendothelium, regardless of the amount of shear stress. GP Ib may also function as a binding site for thrombin (Macintyre 1987).

III–2.3.2 GP V Subunit

This component is leucine-rich and has a molecular weight of 82 kD. It has high affinity for thrombin and an important role in thrombin signalling (Macintyre 1987; Moran 2000). It may have a role in the interaction of platelets with vWF (Modderman 1992). Some studies suggest it is necessary for the functioning of the entire GP Ib–IX complex (Kahn 1999), but it does not seem to be essential for the surface expression of the functional complex (Macintyre 1987; Moran 2000).

III–2.3.3 GP IX Subunit

The GP IX component is a small membrane glycoprotein of just 160 amino acids and a molecular weight of 22 kD. It is a heterodimer of two polypeptide chains (alpha and a beta) linked by disulfide bonds, and consists of a transmembrane domain and a small cytoplasmic tail (Macintyre 1987; Kahn 1999; Moran 2000). It forms a 1:1 non-covalent complex with GP Ib.

III–2.3.4 GP Ib/IX Subunit

The GP Ib/IX subunit has numerous carbohydrate molecules consisting of chains of sialic acid residues. The chains contribute significantly to the negative charge of the platelet surface, attracting enzyme complexes containing tenase and prothrombinase that are important in the coagulation process. Through its interactions with vWF, it promotes adhesion and tethers platelets to blood vessel walls to form a plaque, so they are not detached by shear stress from blood as it flows past (Kahn 1999). In conditions of high shear stress, GP Ib/IX adheres to vWF and the blood vessel subendothelium.

Role in ITP

The GP Ib/IX/V complex contains a number of alloantigens and autoepitopes as well as some major polymorphisms. It is highly immunogenic (Kunicki 1992; Ouwehand 2000) and its target antigens have been extensively studied (Van Leeuwen 1982; Beardsley 1984; Woods 1984; Woods 1984; Tomiyama 1987; Tomiyama 1989). Some studies indicate that GP Ib is a possible target antigen in both drug-induced and autoimmune forms of thrombocytopenia (Stiene-Martin 1998; Kahn 1999; Moran 2000). Increased plasma levels of glycocalicin (which is cleaved from the extracellular region of GP Ib and released into the plasma) are associated with thrombocytopenia caused by platelet destruction-associated, so measurements (the so-called "GC index") may help differentiate immune thrombocytopenia due to increased platelet destruction from that due to impaired production, and it may distinguish different types of ITP (Fujisawa 1993; Stiene-Martin 1998; Wadenvik 1998).

III–2.4 GP Ic/IIa Complex

Structure

The GP Ia/IIa heterodimeric complex is a non-covalent complex of glycoprotein subunits Ic and IIa and is found within the membranes of intact platelets. Its molecular architecture is like that of the IIb/IIIa molecule (see above). It is expressed on a variety of cell types in addition to platelets.

Function

This complex forms the fibronectin receptor. It facilitates adhesion of cells to fibronectin (via the RGD sequence) and the adhesion of inactivated platelets to fibronectin-coated surfaces (Macintyre 1987; Beutler 1995; Stiene-Martin 1998). The Ic subunit consists of disulfide-linked heavy and light chains with *N*-linked oligosaccharides.

Disruption of this receptor will result in disruption in the adhesion of cells to fibronectin and inactivated platelets to fibronectin-coated surfaces.

III–2.5 GP αV/IIIa Complex

Structure

This GP V component contains an alpha chain (αV) which is the CD51 antigen. The IIIa subunit is CD61, which is a 110-kD component composed of heavy chains and kappa light

chains (Modderman 1989a; Modderman 1989b). Between 50 and 100 copies of the complex are expressed on each platelet. It is also found on endothelial cells.

Function

This complex forms the vitronectin receptor. It mediates activation-independent cell adhesion to vitronectin, vWf, fibrinogen and thrombospondin (an extracellular glycoprotein inhibitor of angiogenesis). Adhesion to vitronectin only occurs in the presence of magnesium or manganese. On endothelial cells, the binding of adhesion proteins is inhibited by RGD peptides (Macintyre 1987; Tomiyama 1993; Beutler 1995; Stiene-Martin 1998).Vitronectin-mediated adhesion is likely to be disrupted.

III–3 Other Platelet Antigens and Integrins

The glycoproteins contain various antigenic structures, some of which are involved in immune-mediated thrombocytopenia. Many of these are human platelet antigens (HPAs) which are significant in the immune responses to blood transfusion because they can trigger production of platelet-specific alloantibodies against donor antigens. The glycoprotein complexes GP Ia/IIa, GP Ib/IX/V, GP IIb and GP IIIa contain many HPAs. The thirteen known ones are HPA-1 through to HPA-10, Ly^a, Sit^a, –Gro^a and –Oe^a.

Other antigens on the surface of a platelet may evoke production of antibodies. These include the human leukocyte antigens (HLAs) and the A and B antigens of the ABO blood-typing system.

III–3.1 Human Leukocyte Antigens

The human leukocyte antigen (HLA) system relates to the cell-surface antigen-presenting proteins of the major histocompatibility complex (MHC) in humans). It is important in the immune response and the pathogenesis of certain autoimmune diseases. There are HLA molecules on approximately 73% of platelets in humans (George 1985; Taaning 1992; Daci 1995) expressed on the platelet surface as different classes of MHC molecules. Platelets normally express only class I MHC molecules, where they present fragments of proteins to T cells for attack. Immune responses to the HLA antigens are significant because any disruption will affect T cells. In addition, there is great variety in the amino acid residues of the hypervariable regions of HLA molecules, and any differences in these can affect the development of autoimmune diseases (Janeway 1999).

MHC class I-associated peptides are present on the platelets of healthy people and patients with ITP (Hopkins 2005). Most studies on ITP patients show no change in the frequency of HLA class IA, IB or IC antigens (McMillan 1980; Gaiger 1994).

III–3.2 ABO Blood-Type Antigens

These antigens are most relevant to transfusion reactions and transfusion-induced thrombocytopenia.

III–3.3 Other Integrins

Some of the glycoproteins integrins are very immunogenic and are involved in the pathogenesis of ITP, but there are other types of integrin on the surface of platelets that are important in the immune response and for tissue repair and homeostasis. These integrins mediate attachment between platelets, cells and tissues, as well as transmembrane signalling in both directions (outside–in and inside–out via the OCS), in a process that involves tyrosine phosphorylation and alterations of intracellular calcium and pH (Honda 1998; Tohyama 1998). Because the transfer is in two directions, integrins allow rapid and flexible responses to conditions in the environment. Integrins can switch between different activation states depending on the signalling they are involved in.

All platelet integrins are heterodimeric proteins formed from an alpha (α) and beta (β) subunit. There are at least seventeen α and eight β integrin subunits (Page 1991; Peterson 1998) which occur in at least 24 combinations, providing platelets with sufficient diversity to recognise a range of adhesion molecules (Ruoslahti 1991).

III–4 Platelet Maturation and Function

III–4.1 Development

Platelets are produced in the bone marrow, by budding off from the cytoplasm of megakaryocyte cells. The highly specialised megakaryocyte cells undergo fragmentation in a process known as thrombopoiesis. Megakaryocytes are derived from haematopoietic stem cells by a process known as endoreduplication, in which their DNA is replicated but their

cytoplasm does not change, so the cells mature but do not divide (Branehog 1975). The undeveloped megakaryocytes have a single large nucleus that occupies the majority of the cell volume, but the cytoplasmic content increases during maturation. A smooth internal membrane develops within the megakaryocyte which will form the external membrane of the platelets as they bud off. There are several cycles of endoreduplication before the megakaryocyte breaks down into platelets, leaving a denuded nucleus. A single mature megakaryocyte can produce between 5,000 and 10,000 platelets. Sometimes there are increased levels of bone-marrow megakaryocytes in ITP patients (Semple 1998) but often they are within the normal range.

The regulation of megakaryocyte development and platelet formation takes place at many levels, governed by various cytokines (Kaushansky 1995), but the process of platelet production (known as thrombopoiesis) is stimulated by the glycoprotein hormone known as thrombopoietin. Thrombopoietin (TPO) is a megakaryocyte growth and development factor produced by the liver and the kidney. It regulates the production of platelets by the bone marrow, by stimulating the production and differentiation of megakaryocytes (Kaushansky 1995).

This platelet growth factor influences overall platelet mass (Kaushansky 1995). It is produced in the liver and kidneys and controls the rate of production by pushing haematopoietic stem cells through the various stages of the cell cycle (Ritchie 1996), and it causes the megakaryocytes to undergo proliferation and differentiation. It also reduces apoptosis of megakaryocytes (Ritchie 1996).

The normal range of platelets is 150–450 × 10^9 per litre of blood (that is, 150,000–450,000 cells per μl) of blood (Kuter 1996). Platelet count quantifies platelet production, but other methods include platelet turnover and megakaryocyte mass. Megakaryocyte mass relates the number of platelets in the bone marrow to their volume (calculated from their mean diameter).

III–4.2 Production

Platelet counts in people with ITP are generally low. Generally there will be an increase in megakaryocyte mass, and the relationship between platelet turnover and megakaryocyte mass can indicate the severity of the thrombopoiesis. Platelet turnover is increased in ITP patients, but because some types of platelets may be more resistant to immune destruction, this turnover data cannot be taken at face value.

In the thrombocytopenia seen in ITP patients, some of the reduction in circulating platelets may involve inhibition of platelet development. Current thoughts relate to the impaired production of the platelet growth factor, thrombopoietin. This hormone is responsible for stimulating platelet production, so any defect in its production will directly influence platelet production. Thrombopoietin levels are found to be higher in patients with low levels of platelet production than in those with high levels of peripheral platelet destruction (Porcelijn 1998) and only slightly higher than in healthy people (Kosugi 1996). Because of its potential involvement in the aetiology of platelet depletion disorders, thrombopoietin is now a target for specific drugs called thrombopoietin receptor agonists. Short peptides with the same actions as thrombopoietin may be synthesised chemically. There may also be some

dysfunction of B or T lymphocytes during thrombopoiesis, and possible an increase in activation of mononuclear phagocytes.

Platelet antibodies may also affect the megakaryocytes in the bone marrow. Since megakaryocytes carry the same antigens as mature platelets, platelet antibodies may interact with the megakaryocytes leading to impaired platelet production. In chronic ITP, autoantibodies bind to megakaryocytes (McMillan 1978; Hoffman 1985) and these autoantibodies may cause damage directly to the precursors, in which case platelet development will be affected. They may also disrupt megakaryocyte colony formation and production of lymphocytes (McMillan 1978; Hoffman 1985). However, binding of autoantibodies to megakaryocytes has only been shown *in vitro* .

In some diseases in which there is depletion of platelets in the circulation, which can happen very quickly, the bone marrow may increase platelet production many times over in order to maintain a normal platelet count.

III–4.3 Distribution

There is significant variation in the number of platelets in different parts of the body. However, the total number of platelets in the body usually remains the same (Kuter 1997). About two-thirds, or 70%, of platelets are found within the blood circulation at a concentration of 150–400 x 10^9 per litre. About 30% remain in the spleen circulation. The overall average platelet number is 90 x 10^6 per litre of blood (Kuter 1996).

Circulating levels in the blood can be depleted very quickly, even in healthy people. In fact, an average healthy adult must produce about 2×10^{11} platelets every day to keep the circulating level constant. The bone marrow can increase platelet production five- to ten-fold to achieve this (Majerus, 1994).

Platelets live in the circulation for 5–9 days before being removed by phagocytosis in the reticular endothelial cells of the spleen and liver.

III–5 Role in Haemostasis

Platelets have a complex part to play in haemostasis. When a blood vessel wall is damaged, and the subendothelium is exposed, a series of interactions between the blood vessel wall, plasma proteins and the platelet – particularly the glycoprotein complexes in its membrane – will take place. Ultimately, the haemorrhaging of the injured vessel will be stopped and vascular damage will be limited. Platelets are essential for maintaining haemostasis of the body, by causing bleeding to stop, and this is primarily by clot (thrombus) formation.

People with low blood platelet counts can bleed excessively or there can be bruising or the formation of petechiae as in ITP. Conversely, if the platelet counts are too high blood clots can form easily and can block the blood vessels, resulting in a cerebrovascular accident, myocardial infarction or pulmonary embolism.

III–5.1 Clot Formation

When the endothelial cells on the inner surface of a blood vessel are injured, the subendothelial matrix of the damaged blood vessel is exposed. A number of other subendothelial factors are then exposed to the bloodstream where the platelets are circulating. These factors include collagen, vWF, laminin, fibronectin and tissue factor. When the circulating platelets make contact, they are activated. Activation causes a cascade of reactions that causes the platelets to stick to the site of injury in the blood vessel. This is primarily mediated by the glycoprotein complexes on their surface membranes. The large multimeric glycoprotein, vWF, is a cell adhesion ligand that helps the endothelium bind to collagen. Fibronectin is an extracellular matrix glycoprotein that binds to the receptor proteins known as integrins spanning the outer membrane of platelets. These glycoprotein receptors mediate further reactions that contribute further to platelet activation, adhesion and aggregation. The platelets clump together and form clots, and the glycoproteins on adjacent platelets bind with circulating fibrinogen to strengthen the thrombus (Wilson 2005). The clot ultimately forms a haemostatic plug that stops the flow of blood from the injury site.

III–5.2 Surface Receptors

The membrane of the platelet contains several complexes of glycoproteins and phospholipids, as well as substances that are important for platelet function, such as calcium and an enzyme for producing prostaglandins (White 1994). The glycoproteins act as receptors for platelet activators and mediate adhesion reactions. There are at least nine different glycoproteins (GP Ia, GP Ib, GP Ic, GP IIa, GP IIb, GP IIIa, GP IV, GP V and GP IX) and they are all

essential for maintaining haemostasis and immune responsiveness (Beutler 1995; Stiene-Martin 1998). In ITP, autoantibodies are made against these glycoproteins. The membrane phospholipids attract coagulation proteins and act as the substrate for enzymes that produce thromboxane.

III–5.3 Thromboxane

Thromboxane is a type of lipid known as an eicosanoid. The enzyme thromboxane-A synthase, found in platelets, converts the arachidonic acid derivative, prostaglandin H2, to thromboxane. Two major types – thromboxane A2 and thromboxane B2 – are produced by activated platelets. On activation, there is an increase in the production of phospholipase A2. This causes the formation of TXA2 (Rand 1998), which stimulates activation of more platelets and facilitates platelet aggregation. How they are secreted from platelets is still unclear.

Thromboxane acts by binding to thromboxane receptors and it facilitates platelet aggregation. It activates other platelets by binding with specific sites on their surface. The platelets change shape and intracellular calcium levels increase, inducing protein phosphorylation. Protein kinase C is activated, and induces the expression of the glycoprotein GP IIb/IIIa glycoprotein on the platelet membrane. This is the fibrinogen receptor (Francischetti 2000) and it mediates platelet aggregation is achieved when circulating fibrinogen binds these receptors on adjacent platelets. As a result, a clot forms (thrombosis).

III–5.4 Platelet Activation

When platelets are activated, a host of chemical and structural changes occur that are central to the haemostatic process.

III–5.4.1 Conformational Changes

During platelet activation, the contractile cytoskeleton of the platelet induces the platelet to change from a discoid shape to a stellate shape and form surface projections known as pseudopods (Hoffbrand 2001). The long extensions help structurally in the aggregation of one platelet with another.

Another conformational change occurs in the cytoskeleton through internal contraction and rearrangement of the cytoplasm causes the platelet granules to release their contents. The delta granules in the dense bodies secrete activation factors like the potent platelet activator ADP. The alpha granules secrete the activation factors and adhesion molecules such as platelet factor 4, TGF-β1, platelet-derived growth factor, fibronectin, beta-thromboglobulin, vWF, fibrinogen, factor V and factor XIII. The lambda granules secrete enzymes. Fibrinogen, for example, is released into the OCS and into the bloodstream, where it binds to receptors on adjacent platelets and strengthens the forming clot (Wilson 2005). Fibrinogen is also converted by thrombin into fibrin, a major constituent of clots, which binds to fibronectin. Fibronectin also binds to integrins and collagen. The vitronectin promotes cell adhesion and cell spreading. Secondary aggregation follows as a result (Berndt 2000; Rosado 2001) and consequently more molecules of GP IIb/IIIa are exposed on the platelet membrane, to bind more fibrinogen (Hillery 1991; Law 1996) and stimulate the release of more ADP and hence

activate further molecules of the glycoprotein complex. This inside–outside and outside–inside signalling means the LIBS, fibrinogen and RGD all contribute to the formation of fibrin clots (Frelinger 1990)

When platelets are activated by ADP, the GP IIb/IIIa complex on its surface undergoes a conformational change that causes it to be displayed differently on activated platelets, with the expression of new epitopes that allows fibrinogen to bind with a high affinity (Wagner 1996), and contributes to the formation of a haemostatic plug, or clot. However, if a compound containing or mimicking an RGD sequence links to the complex, it can mask this increased display or instead enhances the display on resting platelets (Marder 1993), thus inhibiting platelet activation (Page 1991).

When platelets are activated by ADP and antiplatelet antibodies, the GP IIb/IIIa complex adopts an active state (Shattil 1985; Berndt 2000). It then expresses new epitopes on its surface, two on the GP IIb subunit and one on the GP IIIa subunit. These ligand-induced binding sites (LIBS) are involved in transmembrane signalling (Honda 1995), and enable the complex to transfer information from the inside of the platelet to the outside (Tohyama 1998), and lead phosphorylation of cytoplasmic proteins in the cytoplasmic domain of the GP IIIa subunit by protein kinase C.

III–5.4.2 Biochemical Changes

During platelet activation, phospholipid molecules are transported to the surface membrane, mediated by an enzyme known as scramblase. These phospholipids provide a negatively charged surface that attracts enzyme complexes that aid in the coagulation process. Two important complexes contain the enzymes tenase and prothrombinase. Tenase is an activating complex made up of tissue factor, factor VII, and calcium. Prothrombinase forms a complex with factor X.

On activation, actin-binding protein within platelets is degraded by endogenous proteins. The actin microfilaments are in the cytoskeleton where they support the cell structure and secure the cytoplasm within its surroundings, thus facilitating signal transduction. The GP Ib subunit of the GP Ib/IX/V complex normally binds to the actin at its carboxy-terminal. When the actin-binding protein is degraded, the GP Ib can then move freely, which facilitates the adhesion of platelets to immobilised vWF and the blood vessel subendothelium.

ADP released from the dense bodies (Rand 1998) can bind with a specific group of receptors on the surface of other platelets and activate even more platelets (Murugappa 2006). There are three receptors for ADP on platelets. One receptor is ionotropic and is involved in the influx of calcium ions. Another is a protein-coupled receptor that is involved in the platelet's shape change and activation of phospholipase C. The third is another protein-coupled receptor that mediates the inhibition of adenylate cyclise (Francischetti 2000).

Finally, during platelet activation, there is an increase in the production of phospholipase A2, which induces the formation of TXA2 (Rand 1998). This stimulates activation of more platelets and facilitates platelet aggregation.

III–5.4.3 Inhibition of Platelet Activation

Platelet activation can be inhibited when the tripeptide RGD links to the GP IIb/IIIa (fibrinogen receptor) complex on the surface of a platelet (Page 1991). In normal haemostasis, it can also be inhibited by substances like nitric oxide, prostacyclin PGI 2 and endothelial-ADPase from the thin endothelial layer inside blood vessels. The enzyme endothelial-ADPase, for example clears away the highly potent platelet activator, ADP (Rand 1998). The eicosanoid prostacyclin PGI 2 directly inhibits platelet activation (Robbins 2010).

III–6 Dysfunction and Destruction

III–6.1 Dysfunction in ITP

Blockade of platelet function can occur during thrombopoiesis, possibly through increased activation of mononuclear phagocytes.

In ITP patients, antiplatelet antibodies cause both platelet destruction and platelet dysfunction. The antiplatelet antibodies formed in ITP are linked with platelet dysfunction. Platelets function abnormally as a result of autoantibodies that recognise antigens on their surface (such as their surface glycoprotein complexes) and bind to FcγRII receptors (Rand 1998). They inhibit platelet adhesion and platelet aggregation (Yanabu 1993; Wadenvik 1998)

examined the sera from ITP patients. They found that the IgG autoantibodies inhibited platelet aggregation when they reacted with a 24-kD membrane glycoprotein (Yanabu 1993). Sera from ITP patients that contains antibodies against the glycoprotein complex GP IIb/IIIa has been found to inhibit the platelet activator, ADP. It also inhibits collagen-induced aggregation.

Some antiplatelet antibodies are also associated with prolonged secondary platelet aggregation (Zahavi 1974). Because the process is prolonged, it will gradually deplete the contents of the platelet granules, such as ADP and ATP, and may cause a storage deficiency (Colman, Kuchibhotla et al. 1977; Yanabu 1991).

The capacity of platelets to form a clot may also be affected, because aggregation in response to ADP and collagen will become blocked, resulting in increased bleeding time. In ITP patients, antibodies against the glycoprotein GP Ib subunit can inhibit the binding of vWF to GP Ib, thus disrupting the clotting response that is started by vWF. Furthermore, the interaction between platelets and collagen will be inhibited (Kornecki 1990; Christie 1992).

There is some controversy about the effect of antiplatelet antibodies on platelet function. Some antibodies isolated from sera of ITP patients have been shown to inhibit platelet aggregation, but similar antibodies from other patients have been shown to activate platelet function (Hashimoto 1994; Taylor 2000). Aggregation assays carried out with antiplatelet IgG isolated from the serum of an ITP patient reacted with glycoproteins with molecular weights of 55 kD and 49 kD, but there was a long delay before aggregation occurred. When antiplatelet antibodies were induced by ADP, aggregation was rapid (Caprino 1974).

III–6.2 Destruction in ITP

Platelets have a mean lifespan of 7–10 days in normal healthy people. They are usually destroyed in the spleen by macrophages, or in the liver by Kupffer cells in the liver in more severe cases (McMillan 2000). Circulating platelets in people with ITP are destroyed by phagocytosis or complement-induced lysis. In conditions with increased peripheral platelet destruction, the survival of platelets ranges from a few days to a matter of minutes. They can be destroyed by antibody-mediated phagocytosis or by lysis. This platelet destruction involves complement activation and binding of antibody to megakaryocytes which decreases platelet production.

ITP patients who have undergone splenectomy may still show thrombocytopenia, so other organs of the reticulendothelial system may be involved, such as the liver (Panzer 1986; Najean 1997).

III–6.2.1 Phagocytosis

Platelets in ITP are destroyed by the mononuclear phagocytic system, although additional mechanisms may be involved in some subsets of ITP patients.

Phagocytosis depends on the formation of specific antibodies. The process is initiated by the Fc regions on the tail of IgG autoantibodies. The Fc region interacts with both the Fc receptors and various proteins of the complement system, such as C3b fixed on the cell surface. The CR1 and CR3 complement receptors and Fc receptors regulate the phagocytic

process (Karpatkin 1992). The Fc receptor called FcyRI has very high affinity for binding IgG.

The IgG-containing antiplatelet antibodies are directed against various glycoprotein receptors in the platelet membrane (Schwartz 2007). In ITP patients, this process may depend on fixation of complement proteins C3, C4 and C9 in ITP (McMillan 2000). The platelets become coated with IgG autoantibodies. When they become coated during this opsonisation process, platelets are marked for ingestion by phagocytosis, usually by macrophages (McMillan 2000). They are recognised by macrophage Fc receptors and may activate complement. Phagocytosis usually begins when the Fc portion of IgG or C3b is fixed to the surface (Karpatkin 1992).

Activation of the complement cascade results in complement-induced lysis, thus the severity of thrombocytopenia in ITP may depend on the ability of antiplatelet antibody to fix complement, as well as the amount of antibodies coating the platelet, and the activity of the Fc receptors

III–6.2.2 Lysis

Lysis describes a type of cell destruction in which the cell is broken down by compromising its integrity, by viral, enzymatic, or osmotic means. The process can be mediated by proteins of the complement cascade. Antibodies fix complement to platelets.

Complement-mediated platelet lysis in ITP has not been well documented clinically, but increased rates of lysis have been observed *in vitro* (McMillan 2000). It may depend on the ability of antiplatelet antibody to fix complement, and determine the severity of thrombocytopenia. Platelet lysis *in vivo* was observed in one with ITP associated with monoclonal IgM antiplatelet antibodies . In the presence of fresh serum, antiplatelet antibodies induced fragmentation and lysis of platelets *in vitro* and generated pro-coagulant platelet microparticles, but this did not happen when the serum was heated, suggesting that complement is involved in this antibody-mediated process . In another study, antibodies specific to epitopes of GP IIIa from a patient with ITP and HIV generated peroxide-induced platelet damage, with platelet fragmentation, procoagulant release, and thrombocytopenia.

Lysis of platelets also leads to the release of glycoproteins from their membranes. This means new epitopes are exposed, and new autoantibodies can be produced against the glycoproteins. A mechanism like this may be important for the pathogenesis of platelet abnormalities in ITP patients.

SECTION IV: ANTIPLATELET ANTIBODIES IN ITP

IV–1 Autoantibody Induction in ITP

ITP is an autoimmune disease in which platelets are self-antigens, stimulating production of antiplatelet antibodies. In ITP patients, the self-antigens are the glycoprotein complexes on the surface of platelets. It has been long-established that this antiplatelet factor is located in the IgG fraction (Shulman 1965), and that IgG is produced by the spleen (McMillan 1974). Patients with ITP show increases in circulating and splenic CD5+ B cells and CD19+ B lymphocytes in both the circulation and the spleen, and the increase correlates with the production of antiplatelet antibodies. Autoreactive T helper cells are significant in the development of autoimmunity, and there is evidence that abnormal activity of T cells stimulates the production of the platelet autoantibodies seen in ITP (Semple 1991; Stasi 2008; Yu 2008). T helper cells also induce lymphocytes to produce various immunoglobulins, including subtypes of the IgG class, namely IgG1, IgG2, IgG3 and IgG4. They initiate somatic mutations in the variable (V) region of genes, which important in the pathogenesis of autoimmune diseases (Janeway 2001).

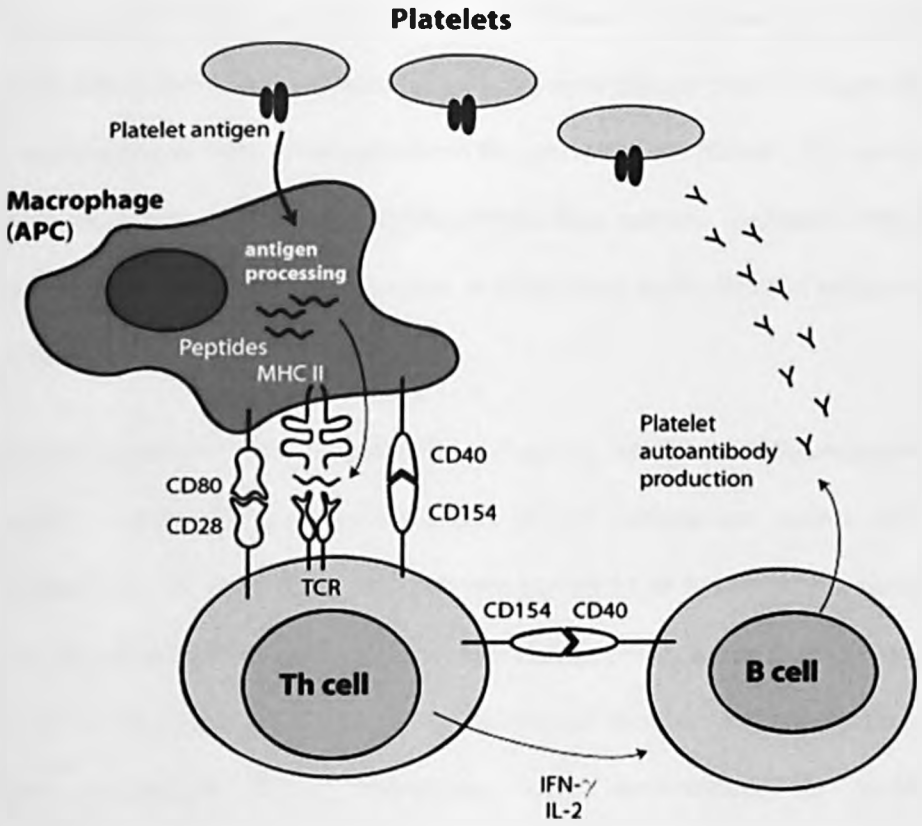


Figure III.8 Emergence of antiplatelet antibodies. Platelet proteins are cleaved into peptides by an antigen-presenting cell (APC) and expressed on the APC cell surface via MHC class II molecules. The T-cell receptor (TCR) of the T helper cell can then bind the peptide–MHC complex and signal activation that upregulates CD154 (CD40 ligand) to interact with CD40 on the APC, so that additional co-stimulatory interactions occur. The activated T helper cell produces cytokines (interleukin-2 and interferon-γ) that promote B-cell differentiation and antibody production. (Beardsley 2006).

In chronic ITP the autoimmune process involves the production of specific antiplatelet antibodies in the spleen and bone marrow. An intravascular platelet antigen forms, either from a self-antigen or from some particle on the surface of the platelet. The autoimmune response is initiated, first in the spleen and then in the bone marrow (Askonas 1958; McMillan 1974; Fujisawa 1993) with the development of antiplatelet antibodies and antigen-specific memory cells.

Antiplatelet antibodies are usually directed against the glycoprotein complexes on the platelet surface. Around 25% of autoantibodies in ITP patients are against different membrane glycoproteins or glycolipids, and they are known to be mostly of the IgG immunoglobulin type (Shulman 1965; Shulman 1965; McMillan 1974). Some of these autoantibodies will attach to the glycoprotein epitopes on the external membrane of the platelets and bring about their destruction. These interactions were demonstrated in various studies of thrombocytopenia and platelet destruction and the enhanced production of platelet-binding IgG in cells from ITP patients (Semple 1998; Wadenvik 1998). IgG-specific autoantibodies can be detected in about 60% of ITP patients (Coopamah 2003).

Other immunoglobulin isotypes, such as IgA and IgM, are also produced in the platelet autoantibodies both the chronic and acute forms of ITP (He 1994). Quantifying these autoantibodies may allow immune thrombocytopenia to be distinguished from thrombocytopenia of non-immune cause (Wadenvik 1998; Winiarski 1998).

The platelet-specific autoantibodies cause both platelet destruction and platelet dysfunction in ITP. Monoclonal antibodies specific for the glycoprotein complex GP IIb/IIIa activate

platelets via the platelet FcγRIIA receptor (Anderson 1991; Rubinstein 1991; Berndt 1993) and lead to increased Fc-receptor-mediated destruction of platelets in the peripheral circulation by macrophages. Antiplatelet antibodies also affect the megakaryocyte precursor cells within the bone marrow, by reacting with antigens on their surface that are also found on mature platelets. This cross-reactivity can cause impaired platelet production. Around 20% of patients with ITP have impaired megakaryocyte function and reduced platelet turnover (Ballem 1987; Nagasawa 1995). People with ITP who have GP Ib-specific antibodies in their serum still have normal numbers of megakaryocytes, but the cells are smaller than those found in patients with GP IIb/IIIa-specific antibodies. It seems likely, therefore, that GP Ib-specific antibodies impair platelet production (Hasegawa 1995). There is also impaired megakaryocyte function in patients with ITP (Ballem 1987), and in the chronic form there is some evidence of autoantibody binding to megakaryocytes.

A weak genetic association has been suggested between autoimmune-mediated thrombocytopenia and alloantigens for platelets. For example, associations have been found between autoimmune thrombocytopenia and the HPA-5a5b genotype in GP Ia, and chronic refractory ITP and HPA-2a (Song 1997).

IV–1.1 T and B Cell Interactions

Various abnormalities of cellular immunity have been reported in chronic ITP. Some antibody-mediated autoimmune diseases develop because of a defect in the T-cell induction of antibodies (Moller 1998), possibly related to the response of T lymphocytes to viral peptides during a viral infection (Kaplan 1992; Moller 1998). T cells are generally thought to

start the production of the autoantibodies, and abnormal T cell activity has been associated with the production of the antiplatelet antibodies seen in ITP (Semple 1991; Stasi 2008; Yu 2008), along with antigen-presenting cells in the early stages. Changes are observed in the ratio of T cells expressing CD4+ and CD8+, with an increase in the proportion of CD8+ cells.

Auto-reactive T helper cells are known to be significant in the development of autoimmunity, and are activated through the binding of antigens to the T-cell receptor (TCR) and the co-receptors CD4, CD3 and CD45 (Janeway 2001). Platelets are the primary source of autoantigens for the stimulation of T helper cells in ITP, and T helper cells have been found to initiate the production of autoantibodies against platelets in the disease (Hedlund-Treutiger 1998). There is a reduction of CD4+ suppressor-inducer cells and an increase in CD4+ helper-inducer cells and HLA DR+-activated T cells (Semple 1998). A decrease in the T-suppressor cell population may be the cause of the diminished AML (autologous mixed lymphocyte) reaction observed in ITP patients.

IV–1.2 T Helper Subtypes and Cytokines

The T helper subtypes Th0, Th1 and Th2 T helper cells produce various cytokines. These include the lymphokine factors, interleukins IL-2, IL-4 and IL-5, interferon gamma (IFNγ) and tumour growth factor beta (TGF-β), In chronic ITP, the activation of Th0 and Th1 cells stimulates an increase in HLA DR^{+}-activated T lymphocytes. This in turn induces production of antiplatelet antibodies from B cells (Semple 1998). The humoral immune response is also affected in ITP patients. During the development of antibodies, T helper cells react with processed antigens on MHC class II molecules that are found on APC cells. Platelet antigens

are processed into smaller peptides fragments within phagolysosomes, before being transferred onto the surface of macrophages with the help from MHC class II molecules (Semple 1998). In ITP patients, peptides from platelet-specific proteins and other general cellular proteins are presented to the immune system via MHC class I molecules. In thrombocytopenia due to increased peripheral platelet destruction, platelets normally only express MHC class I molecules. Various HLA types, responsible for presenting peptide fragments of antigens and self-antigens to T lymphocytes, have been reported to be increased in several studies (Helmerhorst 1982; Gratama 1984; Nomura 1998), but in most patients with ITP there is no change in class I antigens such as HLA-A, HLA-B or HLA-C (McMillan 1980; Gaiger 1994). Any differences in HLA molecules are likely to have an impact on autoimmune responses (Janeway 1999). Although direct correlation has been between glycoprotein-specific autoantibodies for IIb/IIIa and Ib/IX and two DRB1 HLA antigens in ITP patients (Nomura 1999; Kuwana 2001), no strong relation has so far been observed between ITP and the *HLA* genes (Taaning 1992).

When the T lymphocytes recognise the antigen in association with MHC molecules, the B cells are activated. An increase HLA DR^+-activated T lymphocytes induces the production, via Th0 and Th1 cells, of antiplatelet antibodies from B cells (Semple 1998). Another subset of T helper, known as Th2 cells, activate specific B lymphocytes, causing them to proliferate and differentiate into antibody-producing plasma cells. There is cross-linking by antigen of membrane immunoglobulin on the surface of B lymphocytes. Th0 and Th1 cells are activated, stimulating an increase in HLA DR^+-activated T lymphocytes. This in turn induces production of antiplatelet antibodies from B cells (Semple 1998). Increases are observed in

B lymphocytes expressing CD5+ and CD19+ in both the circulation and the spleen, with a significant increase in circulating and splenic CD5+ B cells that correlates with the production of antiplatelet antibodies.

The release of certain cytokine factors from T helper cells is also essential to the activation of B cells. These include the interleukins IL-2, IL-4 and IL-5. The factors IL-4, IFNγ and TGF-β released from Th2 cells induce a switch of the μ chain in B lymphocytes to γ1, γ2, γ3 or γ4 chains. T helper cells also induce lymphocytes to produce various immunoglobulins, including various subtypes of the IgG class, namely IgG1, IgG2, IgG3 and IgG4. They initiate somatic mutations in the variable (V) region of genes, which is a key process in the pathogenesis of autoimmune diseases (Janeway 2001). Approximately 70–85% of patients with ITP or suspected ITP have positive platelet-associated IgG (Christopoulos *et al.*, 1993), and platelet-specific IgG has been detected to GP IIb/IIIa and GP Ib/IX in patients with thrombocytopenia (Brighton 1996).

The autoantibodies in ITP are produced against the epitopes of glycoprotein complexes on the surface of platelets. They bind to platelet FcγRII receptors (Rand 1998) and when coated with IgG autoantibodies, the platelets undergo opsonisation. These glycoprotein complexes are all important in normal haemostasis and clot formation, and the bleeding disorders seen in ITP are caused by disruption of their interactions in haemostasis. Further characterisation of the specific glycoprotein complexes affected in ITP will clarify their role in the disease process.

IV–2 Detection of Antiplatelet Antibodies

There are many assays for detecting autoantibodies and platelet-associated antibodies in serum, with direct assays for platelet-bound immunoglobulins on the surface of intact platelets. Measures of platelet-associated immunoglobulin and glycoprotein-specific antiplatelet antibodies are widely used in investigations of ITP (Mueller-Eckhardt 1989). In antigen capture assays, solubilised platelet antigens are immobilised on plastic wells and are exposed to an antibody. Monoclonal antibodies for specific platelet proteins can help determine the targets of antiplatelet antibodies. The specificity of platelet-associated IgG for different platelet glycoproteins on the platelet may be detected (Berchtold 1993).

IV–2.1 Enzyme-Linked Immunoassay (ELISA)

ELISA is widely used and a range of methods are used. They all involve attachment of a platelet suspension to microtitre wells as a monolayer or detergent lysate antibodies tagged as ligands with peroxidase or alkaline phosphatase. Commercial ELISA kits can be used to detect glycoprotein-specific antibodies in samples of serum. ELISA has been used to detect GP IIIa/IIb autoantibodies on whole platelets and platelet lysate (Brighton 1996) and it has also been used to quantify IgG and demonstrate the role of platelet-associated IgG in the inhibition of platelet aggregation in ITP patients (Yanabu 1993; Hasegawa 1995; Nagasawa 1995).

IV–2.2 Flow Cytometry

Flow cytometry is a technique for counting and analysis of the physical or chemical properties of particles such as cells. Cells are suspended in a stream of fluid and passed through an electronic detection apparatus. The specificity and sensitivity of the method for diagnosis are greatly increased if glycoprotein-specific mouse monoclonal antibody is used. Flow cytometry can be used to detect the young circulating platelets known as reticulated platelets, and platelet-associated IgG in ITP.

IV–2.3 Immunobead assay

An immunobead assay uses plastic beads coated with an antigen or antibody that aggregates or agglutinates in the presence of a specific antibody or antigen. The assay has been used to detect platelet-associated IgG in ITP and GP IV-specific platelet-associated IgG. However, the assay is subject to methodological and practical limitations.

IV–2.4 Immunoblotting

Antiplatelet antibodies can be detected in serum samples by an immunoblotting assay. The technique involves immobilising proteins from a cell culture or tissue sample on special membranes before various monoclonal or polyclonal antibodies are applied (Towbin 1979; Burnette 1981). There are different blotting techniques, such as dot blotting and western blotting. In western blotting, electrophoresis is used to separate out proteins of different

masses on a gel, before transferring them onto a membrane to be probed with specific "marker" antibodies (Burnette 1981).

IV–2.5 Modified Antigen Capture ELISA (MACE)

In the MACE technique, platelets from patients (or control platelets) are added to microtitre plates coated with monoclonal antibodies to capture specific immune complexes of platelet glycoproteins and antibodies. It has been used to detect anti-GP IIIa/IIb autoantibodies on whole platelets and platelet lysate and anti-GP IIb/IIIa and anti-GP Ib/IX platelet-associated IgG in patients with non-immune thrombocytopenia and ITP. It can detect the specificity of platelet-associated IgG for different glycoproteins on platelets (Brighton 1996).

IV–2.6 Monoclonal Antibody Immobilisation of Platelet Antigen (MAIPA)

MAIPA is sensitive, reproducible, assay with a high specificity that gives consistent results (Wadenvik 1998; Winiarski 1998). It is widely used, but it has methodological and practical limitations. For example, it has been known to give false-negative results if there are low platelet counts or low levels of antibody or antibody without specific for the major platelet glycoproteins. The principle involves incubating patient platelets with bound autoantibody (or controls) with mouse monoclonal antibody for a platelet surface antigen. The monoclonal antibody is captured so that the surface glycoprotein–antibody complex can be immobilised and detected using an IgG probe. MAIPA can be used to identify autoantibodies on whole platelets and platelet lysate (Brighton 1996) it is one of the best methods for distinguishing between immune and non-immune forms of the disease. It can detect GP IIb/IIIa-specific

platelet-associated IgG and GP Ib/IX-specific platelet-associated IgG in patients in samples from thrombocytopenic patients with and without an immune basis (Brighton 1996). Glycoprotein-specific platelet-associated IgGs have been shown to occur in significantly different percentages in patients with ITP compared to patients with non-immune thrombocytopenia (Brighton 1996).

IV–2.7 Platelet Immunofluorescence Tests (PIFT)

The method is relatively insensitive, but it is often used for quantifying platelet-associated IgG in the diagnosis of ITP. Platelets from the patient are incubated with fluorescence-labelled anti-IgG, anti IgM and anti-IgG. In the direct PIFT assay, fixed platelet suspensions are incubated with anti-human antibody which it can analysed by flourecence microscope. Platelet-associated IgG, IgM and IgA were detected in 92%, 42% and 9% of patients with ITP, respectively and the technique has been used to show platelet-associated IgG in 70–85% of patients known to have ITP (Christopoulos 1993).

IV–2.8 Platelet Radioactive Antiglobulin Test (PRAT)

In this quantitative antiglobulin consumption assay, radioactive-labelled anti-IgG is bound to IgG on the surface of the platelet. Investigations of ITP often rely on measures of platelet-associated IgG (Panzer 1986).

IV–3 Autoantibodies to Platelet Glycoproteins in ITP

When lysed platelets release glycoproteins from their membranes, autoantibodies are produced against the exposed epitopes of the glycoproteins. Such autoantibodies are found in approximately 60% of ITP patients (Coopamah 2003) and these are mostly specific (more than 90%) for the IgG isotype (Schwartz 2007). The glycoprotein complexes all act as receptors for substances that mediate the haemostatic response, and all are essential in normal clot formation. Disruption therefore leads to disordered haemostasis and the kinds of bleeding disorders seen in ITP. Around 25% of autoantibodies found in ITP patients are for different membrane glycoproteins and glycolipids. The binding of the glycoprotein autoantibodies is not spread widely over the glycoprotein complexes. They attach to limited regions or epitopes on individual glycoprotein subunits, to specific sequences or to the amino or carboxy terminal.

IV–3.1Anti-GP IIb/IIIa Antibodies

IV–3.1.1 Findings in ITP

The majority of antiplatelet antibodies (75%) are made against antigens on the GP IIb/IIIa and GP Ib–IX complexes (Van Leeuwen 1982; Beardsley 1984; Woods 1984). Autoantibodies for the GP IIb/IIIa complex are sometimes measured in high levels and are detected in approximately 15% of patients. Two studies have demonstrated GP IIb/IIIa-specific antibodies in approaching 90% of ITP patients. One patient with a bleeding disorder had

GP IIb/IIIa-specific autoantibodies together with GP IV-specific and GP Ia/IIa-specific autoantibodies (Beer 1993).

Determining whether autoantibodies attach to the GP IIb or GP IIIa subunits is not easy (Yanabu 1991), but the autoantibodies are known attach to limited regions on both of the glycoprotein subunits. (Kosugi 1996) identified as cation-dependent regions of GP IIb/IIIa as autoantigenic targets of platelet-associated IgG.

Binding of anti-GP IIb/IIIa autoantibodies is usually to the GP IIb subunit, at its amino-terminal end. They also bind to epitopes that are close to calcium-binding sites (McMillan 2000).

Sometimes autoantibodies attach to the GP IIIa subunit in the cytoplasmic region of the carboxy-terminal end (Bowditch 1995). GP IIb/IIIa-specific platelet-associated IgG have been shown to bind to the amino acids 35–50 of the GP IIIa subunit. There is some evidence that they attach to cation-dependent epitopes on the GP IIIa component, but only if the whole GP IIb/IIIa complex is conformationally intact. Other proposed targets include a 33-kDa cysteine-rich region, and amino acids 73–39 (McMillan 2000).

IV–3.1.2 Effects of Disruption

The GP IIb/IIIa complex forms the fibrinogen receptor and is involved in the immune response and regulation of haemostasis. It is one of the most studied glycoprotein complexes and one of the most immunogenic, with several known autoepitopes and alloantigens, and some clinically important polymorphisms (Tomiyama 1987; Kunicki 1992; Ouwehand 2000).

Serum from ITP patients with these autoantibodies can inhibit the platelet activator, ADP, as well as collagen-induced aggregation, and there is evidence that GP IIb/IIIa-specific monoclonal antibodies can activate platelets via the platelet FcγRIIA receptor (Anderson 1991; Rubinstein 1991; Berndt 1993), which suggests that antiplatelet antibodies to this glycoprotein complex trigger platelet activation via platelet Fc receptors.

IV–3.2 Anti-GP Ib/IX–V Antibodies

IV–3.2.1 Findings in ITP

Antiplatelet antibodies against the GP Ib–IX complex have been demonstrated in several studies (Van Leeuwen 1982; Beardsley 1984; Woods 1984). Around 75% of antiplatelet antibodies are known to be against antigens on the GP Ib–IX complex. High levels of plasma autoantibodies and platelet-associated antibodies against GP Ib–IX have been found in patients with ITP. Tani, (1989) found GP Ib/IX-specific platelet-associated IgG in 80% of the ITP patients in their study. (McMillan 1987) detected platelet-associated IgG in ITP patients that showed specificity for the GP Ib subunit. Furthermore, a class I MHC-associated peptide was found on the platelets of ITP patients that correlates with an HLA allele and corresponds to the GP Ib unit (Hopkins, 2005). GP IIb/IIIa-specific and GP Ib-specific platelet-associated IgG antibodies were detected in ITP patients by (Hasegawa 1995).

The GP Ib/IX complex is highly immunogenic, with several identified alloantigens and epitopes that have been extensively studied (Van Leeuwen 1982; Beardsley 1984; Woods

1984; Kunicki 1992; Ouwehand 2000). Autoantibodies to GP Ib/IX are known attach only to limited regions, and a very specific region on the GP Ib subunit.

Some studies indicate that GP Ib is the target antigen in autoimmune thrombocytopenia (Stiene-Martin 1998; Kahn 1999; Moran 2000) and platelet-associated IgG with specificity for the GP Ib subunit has been shown in ITP patients (McMillan 1987; Hasegawa 1995). The patients in the study by (Hasegawa 1995) were positive for platelet-associated IgG. Other proposed targets include the one on the GP Ib alpha component, close to the membrane, and extracellular epitopes on the GP Ib beta component (Joutsi-Korhonen 2000). Further associations have been shown in ITP patients with the B7 allele of the HLA system that relates to GP Ib (Hopkins 2005).

Three separate studies (Tani 1989; Kiefel 1996; Hurlimann-Forster 1997) demonstrated antibodies specific for GP Ib/IX in patients with ITP. In one of these studies, they were found to be platelet-associated IgG antibodies (Tani 1989).

IV–3.2.2 Effects of Disruption

GP Ib–IX–V complex forms the vWF receptor. There are approximately 11,000 copies on the platelet membrane (Kahn 1999). Through interactions with vWF, thrombin and actin, the complex facilitates platelet adhesion to vascular subendothelium after vascular injury, and it is involved in processes that lead to platelet activation and thrombus formation. The GP Ib component is thought to be involved in the aetiology of autoimmune thrombocytopenia (Stiene-Martin 1998; Kahn 1999; Moran 2000) and its is associated with the impaired

megakaryocyte production of platelets seen in ITP (Hasegawa 1995). In this study, patients with GP Ib specific-autoantibodies had normal numbers of megakaryocytes, but they were smaller than those found in patients with GP IIb/IIIa-specific antibodies (Hasegawa 1995). Platelet-associated IgG was positive in these patients.

IV–3.3 Anti-GP Ia/IIa Antibodies

IV–3.3.1 Findings in ITP

Circulating antiplatelet antibodies with specificity for the GP Ia/IIa complex have been demonstrated, but there is limited evidence for their presence in patients with ITP. One patient with a bleeding disorder was shown to have GP Ia/IIa-specific antiplatelet antibodies (Godeau 2008).

The GP Ia subunit interacts directly with collagen molecules and platelet aggregation can be blocked by anti-GPIa monoclonal antibodies.

GP Ia/IIa interacts with collagen and platelets lacking GP Ia fail to react properly with collagen of this receptor may have an effect on collagen-induced platelet aggregation. Studies suggest this receptor is involved in type I collagen-induced platelet spreading and aggregation (Monnet 2000).

IV–4 Other Autoantibodies in ITP

IV–4.1 Autoantibodies to Other Platelet Antigens

In the pathogenesis of ITP, intravascular platelets can form self-antigens from some non-glycoprotein particles. Antibody targets other than glycoproteins have been found on platelets in ITP patients. These are various unidentified proteins and glycolipid, glycosphingolipid and phospholipid molecules.

In a meta-analysis, 30% of people with ITP were found to have antiphospholipid antibodies. Circulating antibodies against various internal platelet proteins, such as vinculin, talin, and tropomyosin have also been demonstrated in ITP patients and controls (Tomiyama., 1992). These proteins are all components of the cytoskeleton. However, they are not expressed on the surface of normal platelets, and are only detected in the sera of ITP patients following lytic destruction of platelet, when they are exposed and become autoantigenic (Wadenvik 1998).

Another antigenic target among ITP patients is a platelet granule membrane glycoprotein of 140 kDa, GMP-140. This protein translocates from the secretory granules of platelets and endothelium to the surface after activation (Bierling 1994).

Other antigens on the surface of a platelet may evoke production of antibodies. These include the human leukocyte antigens (HLAs) and the A and B antigens of the ABO blood-typing system. These ABO antigens are associated with transfusion reactions and transfusion-induced thrombocytopenia.

The HLA system relates to the cell-surface antigen-presenting proteins of the MHC in humans. The system is important in the immune response generally and in the pathogenesis of certain autoimmune diseases (Janeway 1999). There are MHC molecules on approximately 73% of platelets in humans (George 1985; Taaning 1992; Daci 1995), normally class I MHC molecules. MHC class I-associated peptides are present on the platelets of healthy people and patients with ITP (Hopkins 2005). Most studies on ITP patients show no change in the frequency of HLA class IA, IB or IC antigens (McMillan 1980; Gaiger 1994).

Human platelet antigens (HPAs) are found within the glycoprotein complexes. They can trigger production of platelet-specific alloantibodies against donor antigens. Platelet HPAs include HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, HPA-6, HPA-7, HPA-8, HPA-9, HPA-10, Ly^a, Sit^a, $-Gro^a$ and $-Oe^a$.

IV–4.2 Autoantibodies to Other Cells

There is experimental evidence of binding of autoantibodies to megakaryocytes in chronic ITP patients (McMillan 1978; Hoffman 1985). . These *in vitro* studies showed that IgG-specific autoantibodies produced by ITP patients bind to megakaryocyte-associated antigens on the precursors. Autoimmune reactions against megakaryocytes, which are associated with platelet dysfunction, arise because the megakaryocytes carry the same antigens as mature platelets on their surface, which antiplatelet antibodies may interact with. Ultimately this disrupts megakaryocyte colony formation and platelet development (McMillan 1978;

Hoffman 1985). The impaired megakaryocyte production of platelets in ITP occurs in approximately 20% of patients with ITP (Ballem 1987) and it is thought to depend on autoantibodies against the glycoprotein GP Ib (Hasegawa 1995). ITP patients with circulating GP Ib-specific autoantibodies had normal numbers of megakaryocytes, but these were smaller in size than those found in patients with GP IIb/IIIa-specific antibodies (Hasegawa 1995).

IV–5 Platelet-Specific Immunoglobulins in ITP

Around 80% of chronic ITP patients have antiplatelet antibodies of the immunoglobulin classes IgG, IgA and IgM against epitopes of glycoproteins on the external membrane of platelets (He 1994). IgM and IgA levels tend to be higher in people with acute ITP, and IgM and IgA and IgG tends to be higher in the chronic form. Antiplatelet antibodies of all three immunoglobulin classes have been reported to correlate with the platelet count (Kokawa 1991; Movahed Shariat Panahi 1994) and therefore they would all be expected to have a role in the pathogenesis of ITP. However, IgM and IgA antiplatelet specific antibodies do not contribute greatly to thrombocytopenia caused by increased platelet destruction, which is the mechanism involved in ITP. (Kiefel 1996) detected glycoprotein-specific platelet-associated immunoglobulins in patients with autoimmune-mediated thrombocytopenia – 46% were IgG specific, 15% were IgM specific and 3% were IgA specific. In another study, platelet-associated IgG was found in 92% of patients with ITP, IgM was found in 42% and IgA in 9% (van der Schoot 1986).

IV–5.1 Platelet-Associated IgG

Numerous autoantibodies with different specificities are found in an individual ITP patient. About a quarter of the autoantibodies produced by ITP patients are against different membrane glycoproteins or glycolipids, and the serum of 80% of chronic ITP patients contains platelet-specific immunoglobulins of various kinds. About 90% of the autoantibodies are known to be of the IgG immunoglobulin type (McMillan 1974; Schwartz 2007) and they can be detected in about 60% of ITP patients (Coopamah 2003). Platelet-associated IgG has been detected on the platelet membrane in 75–90% of patients with ITP (McMillan 1987; Berchtold 1993). The main isotype is IgG (Wadenvik 1998; Winiarski 1998) and splenic IgG production has been shown to be increased in patients with ITP (McMillan 1974).

Unfortunately, the presence of platelet-associated IgG is not specific for ITP (Mueller-Eckhardt ,1980). It is also found in patients with thrombocytopenia from non-immune causes (Helmerhorst 1982) and in patients with other systemic blood diseases. In one particular study, platelet-associated IgG was found in 78% patients with ITP and in 81% patients with non-immune thrombocytopenia (Warner, 1999). Other studies have shown glycoprotein-specific platelet-associated IgG in 46% of patients with autoimmune-mediated thrombocytopenia (Kiefel 1996) and 92% of patients with ITP (van der Schoot 1986).

Measurement of platelet-associated IgG is complicated due to naturally occurring antibodies, internal platelet IgG and aggregated IgG in immune complexes bound to platelets (Rosenfeld 1985; Muniz-Diaz 1995). Normal platelets contain approximately 5 femtograms of IgG, of which only about 1% occurs on the platelet surface. Most is contained within the platelets,

within the alpha granules (Leissinger 1992) and this is mostly IgG1 and IgG3 (Taaning 1988). The amount of platelet-associated IgG is inversely proportional to platelet count and to the mean platelet lifespan (Panzer 1986). In ITP patients, any increase correlates with a reduced platelet count. However, antiplatelet antibodies may be present even if the platelet count is normal.

Antiplatelet IgG has been shown to fix the complement protein C3 to platelets, and this ability to fix complement influences in part the severity of thrombocytopenia (McMillan 2000).

IV–5.2 Glycoprotein-Specific Platelet-Associated IgG

IgG-specific antiplatelet antibodies are directed against various glycoprotein receptors in the platelet membrane (Schwartz 2007). (McMillan 1987) demonstrated glycoprotein-specific platelet-associated IgG in 75% of patients with ITP. (Tani 1989) detected glycoprotein-specific specific platelet-associated IgG in 80% of patients with ITP.

Most of these specific autoantibodies are produced against the highly immunogenic glycoprotein complexes GP IIb/IIIa (Yanabu 1991) and GP Ib–IX (Van Leeuwen 1982; Beardsley 1984; Woods 1984). The major autoantigens are found on the GP IIb/IIIa complex (Yanabu 1991), with anti-GP IIb/IIIa autoantibodies occurring in approximately 15% of ITP patients. These antibodies react with the carboxy-terminal region of GP IIIa, which is located within its cytoplasmic region, as well as targetting epitopes in other areas (McMillan 2000). GP IIb/IIIa-specific platelet-associated IgG in samples from thrombocytopenic patients (Warner 1997).

Platelet-associated IgG can be detected using a range of analytical methods, such as immunobead, MAIPA and MACE. Improving the detection of glycoprotein specificity will improve the accuracy of tests for detecting and classifying thrombocytopenia that has an autoimmune basis. Detection of glycoprotein-specific platelet-associated IgG also improves our understanding of the nature of antiplatelet antibodies in the different types of thrombocytopenia. For example, GP IIb/IIIa- and Gp Ib/IX-specific platelet-associated IgG was detected in 49% of patients with ITP and in 22% of patients with non-immune thrombocytopenia by (Brighton 1996)., and GP IIb/IIIa-specific platelet-associated IgG was detected in 39% of patients with ITP and some with non-immune thrombocytopenia by (Warner 1997).

Binding of anti-IgG autoantibody on the glycoprotein complexes is highly specific. They attach to very specific regions on the GP IIb/IIIa, GP Ib/IX complexes and the GP IIb and GP Ib subunits (Joutsi-Korhonen 2000). The autoepitopes recognised by circulating antibodies are a 65-kDa site on the carboxy terminal and amino acids 23–38 on GP IIIb (alpha). On GP IIIa, autoantibodies bind to a 33 kD-cysteine-rich site, the cytoplasmic carboxy terminal, a region at amino acids 39-73, and the IIb/IIIa complex. In one ITP patient, there was binding to amino acids 35–50 of GP IIIa (Joutsi-Korhonen 2000). GP Ib alpha antibodies bind to close to the membrane and GP Ib beta antibodies bind to extracellular epitopes.

IV–6 Aims of the Study

The aim of this study is to recognise autoantibodies against human platelet antigens, specifically the glycoprotein complexes targeted in the autoimmune disease ITP, such as GP IIIa/IIb and GP Ib/Ix, using platelet lysate and whole platelets from healthy volunteers and a library of antibody Fab fragments derived from splenic B lymphocytes of a patient with ITP. Anti-platelet Fab fragments will be isolated from a phage library (of electrocompetent *Escherichia coli* (K12) and M13 helper phages) by serial biopanning. ELISA will be used to identify the glycoproteins, and dot blotting and western blotting techniques will be used to further characterise the antigens.

IV–6.1 Objectives

- The isolation of anti- platelet antibodies from an ITP patient by transfection using a phage library
- Characterisation of the isolated antibodies through ELISA, and blotting assays.

II– MATERIALS AND METHODS

II–1 Preparation of Washed Whole Platelets

Materials

• Anti-coagulant, acid citrate dextrose (ACD) – 100 mM sodium citrate, 40 mM citric acid, 140 mM dextrose in distilled water

• Isotonic buffer – 10 mM Tris base, 150 mM sodium chloride (NaCl), 1 mM ethylenediamine tetra-acetic acid (EDTA), pH 7.4

• Isotonic citrate buffer – 50 mM sodium citrate, 100 mM NaCl, 140 mM glucose, pH 6.2

Method

60 ml anti-coagulated (ACD) blood, prepared from healthy volunteer (PhD student) was centrifuged at 200 *g* for 20 minutes at room temperature (15-22 ^{0} C). After which the platelet rich plasma (PRP) was removed. 1 µg/ml diluted prostaglandin (1 in 4 in ethanol) was added and the PRP re-centrifuged at 1200 *g* for 12 minutes. After washing the sedimented platelets four times with isotonic citrate buffer, the platelets were resuspended in 1 ml isotonic buffer containing 10% dimethylethyl sulfoxide (DMSO) and aliquoted at a concentration of 10^{9} ml^{-1} and stored at –20 °C. The thawed platelets were washed with isotonic citrate buffer before use.

II–2 Preparation of Platelet Lysate

Materials

• Lysis buffer – 20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4, 0.004 mM phenylmethanesulfonylfluoride (PMSF), 1% Triton X 100

• Phosphate-buffered saline (PBS) – 100 mM sodium hydrogen phosphate ($NaHPO_4$), 150 mM NaCl, 30 mM EDTA, pH 7.4

All standard laboratory regents and chemical were supplied by the Sigma Company Ltd and BDH (Merck Ltd) in the UK.

Method

Purified whole platelets were prepared as described before (*see II.1*). Then 6 ml lysis buffer was added and incubated at 4 °C for 1 hour, after which the lysate was centrifuged at 20,000 *g* for 30 minutes at 4 °C. The supernatant was aliquoted into 0.5 ml tubes and stored at –20 °C.

II–3 Concentration of Lysate Protein

5 ml of the supernatant was concentrated using a centrifugal filter device (10,000 MW cut-off) at 4,000 *g* for 40 minutes. The concentrated platelet proteins were aliquoted into 500-µl Eppendorf tubes and stored at –20 °C.

II–4 Determination of Platelet Protein Concentration

The Bio-Rad DC protein assay is a colourimetric assay for determining protein concentration following detergent solubilisation. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. The amino acids tyrosine, tryptophan, cysteine, and histidine affect the reduction of the Folin reagent by the loss of 1, 2 or 3 oxygen atoms. This produces one or more of several possible reduced species with a characteristic blue colour with maximum absorbance at 750 nm.

Materials

- DC Protein Assay Kits (Bio-Rad Laboratories) including:

  Reagent A – An alkaline copper tartarate solution

  Reagent B – Folin solution

  Reagent S – For samples that used a surfactant reagents such as Triton X-100

- Standard bovine serum albumin(BSA)

Method

BSA at an initial concentration of 1.6 mg/ml was used to produce a standard curve by serial dilution. A standard concentration range (100 μg/ml, 200 μg/ml, 400 μg/ml, 600 μg/ml, 800 μg/ml, 1200 μg/ml1400 μg/ml, 1600 μg/ml) was prepared with isotonic buffer and two undiluted samples and two samples diluted 1 in 10 with lysis buffer. Working reagent A was

prepared by adding 20 µl of reagent S to 1 ml reagent A. Then 5 µl of each standard and sample along with 5 µl of buffer as a blank and 25 µl of reagent A were added to separate wells of an ELISA microplate (Maxisorb^{TM}).

The plate was shaken carefully. Then 200 µl of reagent B was added to each well and mixed immediately. The plate was left at room temperature for 15 minutes after which the absorbance at 750 nm was read with an ELISA plate reader (MRXII Dynex Technologies).

II–5 ELISA Detection of Platelet Membrane Glycoproteins

Materials

• Monoclonal antibodies anti human CD41 (GP IIb/IIIa) and anti human CD-61 (GP IIIa) (Novacastra Company Ltd)

• Rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody (Sigma Chemical Company)

• Coating buffer – 1M sodium hydrogen carbonate, pH 8.3

• Washing buffer – 0.05% Tween-20 in PBS

• Substrate solution – one 10 mg tablet *o*-phenylenediamine dihydrochloride (OPD) dissolved in 25 ml citrate buffer with 10 µl of 30% H_2O_2 (OPD and H_2O_2 obtained from the Sigma Chemical Company)

Method

Microtitre plate wells were coated with 100 µl of platelet lysate (200 µg/ml) or 100 µl containing 10^7 washed whole platelets and incubated at 4 °C overnight. After washing twice with 250 µl PBS-Tween 20, the plates were blocked with 200 µl of 5% dried milk in PBS and left for 1 hour at room temperature. After two further washes with PBS-Tween 20, 50 µl of mouse monoclonal anti- IIIa (CD61) or anti- IIIa/IIb (CD41), diluted 1 in 100 with 5% dried milk in PBS, was added and incubated for 2 hours at 37 °C.

The plate was washed a further six times with PBS-Tween 20. Then 50 µl of a 1 in 1000 dilution of rabbit anti-mouse IgG HRP-conjugated antibody in 5% dried milk in PBS was added to each well. The plate was incubated for 1 hour at 37 °C. After another six washes with PBS-Tween 20, 200 µl OPD substrate buffer was added to each well. The plate was covered with foil and incubated for 30 minutes at room temperature. The absorbance was read at 450 nm.

I–6 Titration of Anti-Glycoprotein Antibodies

100 µl diluted monoclonal antibody against CD41 over the range 1.0×10^{-2} to 6.25×10^{-4} µg/ml was added to the well of a microtitre plate and incubated overnight at 4 °C. The following morning the plate was washed twice with wash buffer PBS-Tween 20 and blocked with 5% dried milk in PBS for 2 hours at 37 °C. The plate was washed once, as above. 100 µl

of washed platelets containing 10^7 platelets was added to each well and incubated for 2 hours at 37 °C in a carbon dioxide incubator.

The anti-platelet antibodies were detected by the addition of 100 µl rabbit anti-mouse IgG HRP-conjugated antibodies diluted 1 in 1000 with 5% dried milk in PBS. The plate was then incubated for 2 hours at 37 °C. After six more washes, 200 µl OPD substrate was added to each well and left at room temperature for 30 minutes. The absorbance was read at 490 nm.

II–7 Titration of Platelet glycoprotein(CD-41 and CD-61)

100 µl of whole platelet suspension over the range 10^7–10^4 platelets per well or 100 µl platelet lysate concentration over the range 600–100g/ml was added to a microtitre plate and incubated at 4 °C overnight. The following morning the plates were washed twice with PBS-Tween 20, blocked with 5% dried milk in PBS and left for 2 hours at 37 °C. Then 100 µl of mouse anti-CD61 or anti-CD41 at a concentration of 5 µg/ml (diluted with 5% dried milk in PBS) was added and incubated for 2 hours at 37 °C. After six washes with wash buffer, 100 µl of rabbit anti-mouse IgG HRP-conjugated antibody, diluted 1 in 1000 (with 5% dried milk in PBS)was added to each well and incubated for 2 hours at 37 °C. The plates were washed more six times and 200 µl OPD substrate buffer was added to each well. After 30 minutes incubation at room temperature the absorbance was read at 490 nm.

II–8 Preparation of Electrocompetent *Escherichia coli* (K12)

Materials

- Luria broth (LB) 1 L – 10 g tryptone, 5 g yeast extract, 10 g NaCl, made up to 1 L in distilled water, pH 7.0 (adjusted with NaOH)
- LB agar – 1 L of Luria broth, 10 g bacteriological agar
- Super broth (SB) 1 L – 10 g 3-(*N*-morpholino) propanesulfonic acid (MOPS), 20 g yeast extract, 30 g tryptone ,made up to 1 L in distilled water, pH 7.0 (adjusted with NaOH)

Method

2 µl of *Escherichia coli* (K12) from a glycerol stock was cultured on a dry, warm Luria media agar plate and incubated at 37 °C for 24 hours. A single colony was inoculated into 10 ml SB with 15 mg/ml tetracycline and incubated overnight at 37 °C at 200 r.p.m. in an orbital shaker. Then 2.5 ml of the overnight culture of *Escherichia coli* (K12) suspension was transferred into 500 ml SB with 10 ml 20% (w/v) glucose and 5 ml $MgCl_2$, without any antibiotic. The culture was incubated at 37 °C on an orbital shaker until the OD at 600 nm reached 0.7–0.8. After chilling the culture on ice for 15 minutes, it was centrifuged at 3,000 *g* for 20 minutes at 4 °C. The supernatant was then discarded and the cells resuspended in 250 ml chilled wash buffer containing 10% glycerol, and centrifuged at 3000 *g* for 20 minutes. The supernatant was poured off and the washing step was repeated. Then the supernatant was discarded and the cells were resuspended in 12 ml 10% glycerol and transferred to a chilled 500-ml tube.

This sample was centrifuged at 2,300 *g* for 15 minutes at 4 °C. The supernatant was carefully poured off and the cells were resuspended in 3 ml of the wash buffer. The cells were dispensed immediately into 0.5-ml tubes and stored at –80 °C.

II–9 M13 Helper Phage

II–9.1 Preparation of M13 Helper Phage

A colony of *Escherichia coli* (K12) was transferred from LB agar containing 15 μg/ml tetracycline to 10 ml LB broth with supplements (1% 1 M $MgSO_4$ and 0.3% 2 M maltose) and was shaken at 200 r.p.m. at 37 °C. After the optical density (OD) at 600 nm reached 0.3, the commercial helper phage (VCSM13; Stratagene) was added at a multiplicity of infection of 20 to 1 (phage to cell ratio) and was incubated at 37 °C for 15 minutes. Shaking was continued at 37 °C for 30 minutes. Then 25 μg/ml kanamycin was added and the shaking continued for another 7.5 hours. The culture was heated at 65 °C for 15 min in hot water before being centrifuged at 2,300 *g* for 15 minutes. The supernatant was kept at 4 °C.

II–9.2 Titration of Helper Phage

Materials

- NZY agar – 5 g NaCl, 2 g $MgSO_4$ (7 H_2O), 5 g yeast extract, 10 g NZ amine, 15 g agar, made up to 1 L in distilled water, pH 7.5 (adjusted with NaOH)

- NZY top agar – 1 L NZY broth, 0.7% (w/v) agar

- SM buffer – 5.8 g NaCl, 2.0 g $MgSO_4$, 50 ml 1 M Tris-HCl (pH 7.5), 5 ml 2% (w/v) gelatin, made up to 1 L in distilled water

Method

A colony of isolated *Escherichia coli* (K12) (on a tetracycline plate) was transferred into 10 ml LB broth with supplements in a 50-ml conical tube and incubated at 37 °C with shaking, until an OD 600 nm of 1.0 was reached. A serial dilution of the bacteriophage in SM buffer to create 10^{-6}, 10^{-7} and 10^{-8} dilutions of the phage was prepared. 1 µl of each dilution was added to 200 µl of the K12 culture. After incubating the samples for 15 minutes at 37 °C, 3 ml NZY top agar, melted and cooled to 48 °C, was added to the samples, then plated onto dry and pre-warmed NZY agar plates and left to set before being inverted and incubated overnight at 37 °C. The count of phage in the plate was calculated from following equation:

$$\text{Count/ml} = \frac{\text{Number of plaques (p.f.u.) x dilution factor}}{\text{Volume plated (µl)}} \times 1000$$

II–10 DNA Isolation

Alkaline lysis releases plasmid DNA from bacteria and ribonuclease A (RNase A) removes all the RNA in the lysate. In the presence of the chaotropic salt, guanidine hydrochloride, plasmid DNA binds selectively to special glass fibres pre-packed in the HighPure™ filter tube. Bound plasmid DNA is purified in a series of rapid "wash and spin" steps to remove contaminating bacterial components. Low salt elution then releases the DNA from the glass fibre. This eliminates the need for organic solvent extractions and DNA precipitation, allowing for rapid purification of many samples simultaneously.

II–10.1 DNA Preparation

Materials

- HighPure™ plasmid isolation kit available from Roche, with 50 HighPure™ filter tubes (polypropylene tubes with two layers of glass fibre)
- Suspension buffer – 0.05 M Tris–HCl and 0.01 M EDTA, pH 8 (25 °C)
- RNAse A
- Lysis solution – 0.2 M NaOH, 1% sodium dodecyl sulphate (SDS)
- Binding buffer – 4 M guanidine hydrochloride, 0.05 M potassium acetate, pH 4.2
- Wash buffer I –5 M guanidine hydrochloride, 20 mM Tris–HCl, pH 6.6, 75% (v/v) ethanol
- Wash buffer II – 40 ml 0.02 M NaCl, 0.02 M Tris–HCl, pH 7.5

• Elution buffer – 0.01 M Tris–HCl, pH 8.5

Method

100 µl of electrocompetent *Escherichia coli* cells (K12) were inoculated into a pre-chilled tube with 5 µl of plasmid DNA (from a phage library containing DNA of the heavy chain domains VH and CH1, and light chain domains VL and CL, of the antibodies in PAK100 vector, constructed using mRNA obtained from splenic lymphocytes of one patient with idiopathic thrombocytopenic purpura (ITP) and systemic lupus erythematosus (the phage library was a gift from Dr Lynda Partridge at the University of Sheffield).

The sample was left in ice for 2 minutes. Then it was transferred into a pre-chilled electroporation cuvette. A 5-second pulse of 2.5 kV was applied to the sample by a gene pulser (BioRad). Immediately 1 ml SB was added to the cuvette and the contents were transferred to a 15 ml tube. After shaking for 1 hour at 37 °C and 200 r.p.m., 200 µl of the culture was inoculated into 10 ml SB containing chloramphenicol (25 µg/ml) and shaken overnight at 37 °C and 200 r.p.m. The following day the sample was centrifuged at 3000 *g* at room temperature for 15 minutes and the supernatant was discarded. The pellet was treated with a QIA-Prep Spin MiniPrep kit which was designed for the purification of 20 µg of high-copy plasmid DNA.

II–10.2 DNA Loading on Agarose Gel

A 1% agarose gel in TAE buffer (10 mM Tris, 1 mM EDTA) was prepared and left at room temperature for 30 minutes. After this time, 5 µl of the sample, which was diluted with loading buffer(bromophenol blue), 4 ml DNA to 1 ml loading buffer, and 5 µl of DNA marker were inoculated in separate wells. The gel was run at a constant voltage of 100 volts for approximately 40 minutes at room temperature. After running, the gel was visualised and photographed using the UVI Save Imaging System (UVItec Company).

II–10.3 Restriction Enzyme Digestion of DNA

Materials

- Elution buffer – 0.01 M Tris–HCl, pH 8.5
- Restriction enzymes – BstXI , Xhol XI, XbaI , SacI, NehI (Boehringer Mannheim)
- Buffer H – (Boehringer Mannheim)
- Buffer A – (Boehringer Mannheim)
- Buffer M – (Boehringer Mannheim)
- Deionised water

Method

To confirm whether the DNA of the heavy and light chains had been inserted into the plasmid DNA, the DNA was cut with restriction enzymes to isolate the insertion. For each sample, three Eppendorf tubes were prepared: one for the heavy chain digest, one for the light chain digest, and one for the undigested DNA. Then 25 µl DNA was added to each tube, along with 2 µl of the restriction enzymes.BstXI (10 U/µ1) and Xhol XI (10 U/µl) were used for the heavy chain, 2 µl of XbaI (10 U/µl) and 2 µl SacI (10 U µl-1) were used for the light chain, and 2 µl of NehI (10 U/µl) was used for the undigested sample.

II-11 Biopanning

II–11.1 Transfection and Amplification of *Escherichia coli* (K12) by library Phage

50 µl of electrocompetent cells (K12) were placed in a pre-chilled tube with 5 µl plasmid DNA from phage library that contained DNA of the heavy chain (VH and CH1) and light chain (VL and CL) domains of the antibodies. The solution was left on ice for 2 minutes. Then an electric pulse of 2.5 kV was applied by gene pulser for 5 seconds. The solution was washed immediately with LB and transferred to a 50 ml tube. Then 4 ml LB was added and the sample was shaken for 1 hour at 37 °C and 200 r.p.m. after which 10 ml LB containing 25 µg/ml chloramphenicol and 15 µg/ml tetracycline was added to the solution. To check the success of transfection, aliquots of 1 µl, 10 µl and 100 µl of the diluted culture (1 in 100) were plated onto LB agar containing 25 µg/ml chloramphenicol and incubated at 37 °C for 24 hours. Then 5 µl of 25 µg/ml chloramphenicol was added to the sample and shaken for 1 hour. On completion, 2 ml VCSM (helper phage) was added and left for 15 minutes at room temperature. Then 200 ml LB containing 15 µg/ml tetracycline and 25 µg/ml chloramphenicol was added and shaken at 200 r.p.m. for another 2 hours. The solution was then mixed with 70 µg/ml kanamycin and shaken overnight.

The next day, the suspension was centrifuged at 3000 *g* at 4 °C for 15 minutes. The supernatant was transferred to a clean sterile bottle containing 4% (w/v) PEG-8000 and 3% (w/v) NaCl and placed on ice for 30 minutes. It was centrifuged at 15,000 *g* for 20 minutes at 4 °C. The supernatant was discarded, and a paper towel was used to remove as much PEG-

8000 solution as possible. The pellet was resuspended in 2 ml 1% BSA and centrifuged for 5 minutes at 13,000 *g*. The supernatant was stored at 4 °C.

II–11.2 Panning of Platelet Binding Phage

Materials

- Coating buffer- 1M Bicarbonate buffer, pH 8.3
- Washing buffer- PBS-Tween 20 (0.05%)
- Acidic elution buffer- 100 mM glycine- HCl, pH2.2
- Neutralisation solution - 2 mM Tris base

Methods

An ELISA plate was coated with 100 µl of the platelet suspension containing 10^8 platelets in bicarbonate buffer (pH 8.6) and incubated overnight at 4 °C. The plate was washed twice with PBS and blocked with 200 µl of 5% dried milk in PBS. Then it was incubated for 1 hour at 37 °C. After two more washes with washing buffer, 50 µl of the phage suspension (from 10.1) was added to each well and the plate incubated for 2 hours at 37 °C. The excess phage was removed by discarding the liquid phase. The wells were filled with PBS-Tween 20 (0.05%) and pipetted vigorously. After 5 minutes the PBS-Tween 20 was removed. For the first round of panning, the plate was washed once, with PBS-Tween 20. The next four rounds of panning involved five washes.

The phage was eluted by adding 50 µl of acidic elution buffer per well. After leaving at room temperature for 10 minutes it was pipetted vigorously. Then the eluted phage was removed and neutralised with 3 µl neutralisation solution (2 M Tris base).

II–11.3 Library Phage Replication and Re-Amplification

The solution obtained from the last round of panning was inoculated with 10 ml LB containing 2 µl *Escherichia coli* (K12) and 15 µg/ml tetracycline. This was shaken at 200 r.p.m. at 37 °C. Optical density was continuously checked at 600 nm. Shaking was discontinued when an OD of 1 was obtained. The phage was added to the culture and left for 15 minutes at room temperature. After that time, 25 µg/ml chloramphenicol was added and the solution was transferred to 200 ml LB. Shaking continued for another 1 hours. To titre the phage-infected bacteria, 1 µl and 10 µl of the diluted culture (1 in 100) was plated onto LB agar containing 25 µg/ml chloramphenicol. This was incubated at 37 °C overnight.

Subsequently, 2 ml VCSM (helper phage) was mixed with the solution and incubated for 15 minutes at room temperature. Then 25 µg/ml chloramphenicol and 15 µg/ml tetracycline were added and shaking was carried out for 2 hours. After this time, 70 µg/ml kanamycin was added, then shaking was continued overnight at 200 r.p.m. and 37 °C.

The following day, the solution was centrifuged at 3000 *g* and 4 °C for 15 minutes. The supernatant was transferred to a clean 500-ml centrifuge tube containing 4% (w/v) PEG-8000 and 3% (w/v) NaCl. The solution was incubated on ice for 30 minutes and the supernatant

was discarded after centrifugation at 15000 *g* and 4 °C for 15 minutes. The bottle was drained by inverting it onto a paper towel for at least 10 minutes.

The phage-plate was resuspended in 2 ml 1% (w/v) BSA in PBS and transferred to a microcentrifuge tube. The suspension was centrifuged at 15,000 *g* for 5 minutes. Five rounds of panning were carried out as before. The eluted phage was titred by infecting 100 µl of *Escherichia coli,*K12, (OD 0.6 at 600 nm) with 1 µl of 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9} diluted phage with LB broth) for 15 minutes at room temperature. Each dilution was next cultured on an LB plate containing 25 µg/ml chloramphenicol and incubated at 37 °C for 48 hours.

II–11.4 Isolating the Individual Phage from the Phage Pool

After five rounds of panning, the individual colonies isolated from the phage pool were examined for reactivity with platelet proteins using the whole-platelet ELISA technique. Each random colony from the last round of panning was inoculated in 2 ml LB containing 25 µg/ml chloramphenicol and was shaken at 200 r.p.m, and 37 °C. The optical density was continuously checked at 600 nm. Shaking was discontinued as soon as the OD reached 1.0. Then 0.5 mM IPTG was added to each tube before shaking was carried out overnight at 200 r.p.m. and 37 °C. The following day, the solutions were centrifuged at 2800 *g* and 4 °C for 15 minutes. The supernatants were transferred to a clean tube and stored at –20 °C.

II–12 Protein Blotting

Protein blotting involves immobilising proteins on membranes before detection using monoclonal or polyclonal antibodies. Enhanced chemiluminescence (ECL) is used in this method. ECL is a common technique for a variety of detection assays for detecting the horseradish peroxidise (HRP) enzyme, which is tethered to the molecule of interest usually through labelling an immunoglobulin that specifically recognises the molecule.

There are different blotting protocols such as dot blotting and western blotting (Burnette, 1981, Towbin, 1979).

II–12.1 Dot Blotting of Platelet Proteins

Protein detection using the dot blotting protocol is similar to western blotting in that both methods allow for the identification and analysis of proteins of interest. Dot blotting methodology differs from western blotting techniques in that the electrophoresis separation in not performed. Sample proteins are instead spotted onto membranes and hybridised with an antibody probe. Semiquantitative measurements can be made of the spots. It can identify the presence of a target protein molecule, but gives no information about its molecular weight or other properties.. If other molecules of are detected, they will still appear as a single dot.

Materials

- Luminol stock – 250 mM (0.886 g) of luminol in 20 ml dimethylsulfoxide (DMSO) (Sigma Chemical Company)

- P-coumaric acid stock – 90 mM of coumaric acid in dimethylsulfoxide.

- Solution 1- 1 ml luminal stock, 0.44 ml P-coumaric acid stock, 10 ml 1 M Tris base at pH 8.5 made up to 100 ml with distilled water.

- Solution 2- 64 ml of 30% H_2O_2, 10 ml 1 M Tris base (pH 8.5) made up to 100 ml with distilled water

- Final solution – 1 to 1 mixture of solutions 1 and 2

- Coomassie blue stain – 125 ml distilled water, 25 ml glacial acetic acid, 0.1% (w/v) Coomassie Blue, made up to 250 ml with 100 ml methanol, and filtered before used.

- Substrate solution buffer (phosphate citrate buffer) – 25.7 ml (200 mmol) sodium dihydrogen phosphate, 24.3 ml (100 mmol) citric acid, in 50 ml deionised water, adjusted to pH 5.

- Substrate solution – one 10 mg tablet *o*-phenylenediamine hydrochloride (OPD) dissolved in 25 ml citrate buffer, 10 µl 30% H_2O_2 (OPD and H_2O_2 from Sigma Chemical Company)

- Stop solution – 3M HCl

Methods

2 µl of platelet lysate was pipetted onto a membrane and left to air dry. The membrane was incubated in 5% dried milk in PBS at room temperature and shaken or rotated for 1 hour. The

blocking solution was removed and the membrane was incubated with the phage as a primary antibody and shaken for 2 hours at room temperature. The solution was washed with 0.05% PBS-T three times, each for 10 minutes, and was incubated with HRP-conjugated anti-M13 antibody diluted 1 in 1000 in blocking solution for 1 hour. The sample was washed as same above. The blot was developed with 2 ml ECL solution, then washed and placed in a case together with a sheet of photographic film and sealed carefully. The film was placed into developer until blotting dots appeared, at which point it was immersed in water for 30 seconds, and placed into FC40 fixative solution until the bands became clear. If no dots appeared that means there is none of the target protein present in the sample.

II–12.2 Western blotting of Platelet Proteins

Western blotting is a molecular method to detect protein in a given sample of tissue or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are transferred out of the gel and onto a membrane, where they are probed using antibodies specific to the protein. As a result, western blotting can determine the molecular weight of the protein by comparing the movement of the protein on the gel with that of the specified proteins. It can examine the number of proteins in a given sample determine the molecular weight of those protein by comparing several bands of the molecular weight marker (Burnette, 1981, Renart, 1979).

Materials

- Buffer 1 – 1.5 M Tris base, 0.4% (w/v) SDS dissolved 1 in 10 in distilled water, pH 8.4.
- Buffer 2 – 0.5 M Tris base, 0.4% (w/v) SDS dissolved 1 in 10 in distilled water, pH 6.8 .
- Acrylamide gel – 29.2% (w/v) acrylamide gel (Gibco BRL), 0.8% (w/v) bis-acrylamide (Sigma Company) in 70 ml distilled water and filtered through Whatman paper into a dark bottle and stored at 4 °C.
- Resolving gel 8% – 11.5 ml distilled water, 6.5 ml buffer 1, 6.7 ml acrylamide, 15 µl tetramethylethylene diamine (TEMED; Sigma Chemical Company), 250 ml Ammonium perssulphate (APS)10%

APS – should be made up fresh each day (100 mg/ml).

- Stacking gel 3% – 4.87 ml distilled water, 1.87 ml buffer 2, 0.75 ml acrylamide, 10µl TEMED, 75 µl APS 10%.
- Running buffer – 0.3% Tris base, 1.44% glycine, 1% SDS dissolved in 1 L distilled water.
- Blotting buffer – 0.3% Tris, 1.41% glycine, 20% methanol in 800 ml distilled water
- Working sample buffer

100 ml SDS 20%, 100-ml glycerol, 50 ml TEP, 70 mg Bromophenol Blue (BPB)

For the reducing sample buffer 0.155 g of dithiotheritol (DTT) was added to 20 ml of sample buffer.

- Reducing sample buffer – 0.155 g dithiotheritol (DTT), 20 ml sample buffer
- Molecular-weight marker (Gibco BRL)
- pure GPIIb/IIIa(Enzyme Research Laboratories,ERL)

Method

The sample was prepared by diluting one part of sample buffer to three parts of either platelet lysate protein from section II-2 (200 μg/ml) or pure GPIIb/IIIa (8 μg/ml) and boiling for 2 minutes.

The resolving gel at 8% was poured and allowed to set. Then the stacking gel was added. Then 15 μl of the protein sample or 5 μl of molecular weight marker was added to separate wells. The gel was run at a constant voltage of 100 volts for 90 minutes.

The protein was transferred from the polyacrylamide gel to a 0.45-μm nitrocellulose membrane at 100 volts for 90 minutes. Nitrocellulose membrane strips containing the separated platelet proteins were blocked with 5% dried milk protein in PBS for 2 hours at room temperature. Then 3 ml of the eluted phage at 10^6 cfu. (colony forming units) per ml was placed onto separate sample strips.

The negative control was 3 ml of helper phage (M13) at the same concentration (10^6 cfu per ml), and the positive control was 3 ml mouse anti-GPIIIa antibody (1 μg/ml) functioned as the positive control. All samples and controls were incubated overnight at 4 °C.

On the following day, the strips were washed with PBS 0.05% Tween-20 for 2 hours. The washing buffer was renewed every 20 minutes. Then 3 ml of rabbit anti-M13 phage HRP-conjugated antibody was diluted 1 in 1000 with 5% dried milk protein in PBS and added to the wells of all the samples. For the positive control, 3 ml anti-mouse conjugated antibody diluted 1 in 1000 with 5% dried milk protein in PBS was added.

After incubation at room temperature for 1 hour, the strips were washed as described above. The blot was developed with 2 ml ECL solution for 2 minutes, and washed and placed in a case along with a sheet of photographic film and sealed carefully for 5 minutes.

The film was placed into 50 ml of CD18 developer solution (Sigma Aldrich Company) diluted in 200 ml distilled water, until the blotting bands appeared. When they appeared, the film was immersed in water for 30 seconds, then placed into 50 ml FC40 fixative diluted in 200 ml distilled water until the bands were clear. The film was washed with water and left to dry.

SECTION III: Results

III–1 Combinatorial Library Construction

The use of the polymerase chain reaction (PCR) to produce a large number of identical copies of DNA sequences is useful for amplifying the gene segments encoding the V domains of antibody(Saiki 1985). The gene to be replicated is inserted into copies of a plasmid containing genes that make cells resistant to particular antibiotics and a multiple cloning site. The plasmids are then inserted into bacteria by a process called transformation. When the bacteria are exposed to particular antibiotics, only the bacteria that take up copies of the plasmid survive, because the plasmid makes them resistant. The protecting genes are expressed (used to make a protein) and the expressed protein breaks down the antibiotics. In this way the antibiotics act as a filter to select only the modified bacteria(Cheng 1994).

These bacteria can be grown in large amounts, then harvested and lysed (often using the alkaline lysis method) to isolate the plasmid of interest. Antibody specific for a particular antigen can be selected from the library by panning. The phage that binds is recovered and amplified through used to infect fresh *Escherichia coli.*

A single-chain variable fragment (scFv, $5\times10^{5)}$ phagemid library of human immunologlobulin light (L) and heavy (H) chains was constructed in this study. The mRNA from the splenic lymphocytes of a patient with ITP in the vector PAK 100 was used (Krebber 1997). This patient suffered from both SLE and ITP. The phage library was a gift from Dr Lynda Partridge from the University of Sheffield. Glycoproteins that recognise the phage can be

isolated by sequential panning of the library with whole platelets. The phage display system enables the production of large quantities of human Fab fragments.

III–2 Development of an Assay to Detect Antiplatelet Antibodies and Fab

The purpose of this study was to optimise a simple, quick and sensitive ELISA in order to identify and quantify antiplatelet antibodies. The sensitivity of the antiplatelet ELISA technique is high. It can detect platelet-specific antibodies even in patients who have high levels of multi-specific antibodies.

An ELISA was established to determine the binding of antibody to the platelet glycoprotein complexes GP IIb (CD41) and GP IIIa (CD61). No reaction was observed with anti-CD61, therefore only anti-CD41 was used throughout this study. In order to identify and quantify the antiplatelet antibodies, whole washed platelets and frozen platelet lysate were coated on a microplate. These cell preparations were obtained from healthy volunteers (PhD students at the university).

III-2.1 Effect of Different Blocking Buffers in the Glycoprotein ELISA

Assays were undertaken to establish the most appropriate blocking buffer for use in the ELISA and for causing substantial blocking in subsequent experiments. In one experiment, 100 µl of platelet preparation (the equivalent of 10^7 whole platelets) in a bicarbonate buffer was coated onto an ELISA plate, and then a protein was added to block the remaining binding sites on the plate. The effects on the ELISA of three different blocking buffers were investigated. These were 5% bovine serum albumin (BSA), 1% BSA, and 5% dried milk in PBS.

Fig. III-1 shows the results obtained with the three different blocking buffers. All three gave a high absorbance for the anti-GP IIb antibody (CD41) and good discrimination between the sample and the negative control (C.Neg). However, the BSA 5% and dried milk 5% were both slightly better at blocking the non-specific binding compared with the BSA 1%, and the dried milk 5% was marginally better than the BSA 5%. Therefore, dried milk at 5% in PBS was used in all subsequent ELISA assays.

Figure III–1 Effect of blocking buffer on ELISA detection of human CD-41

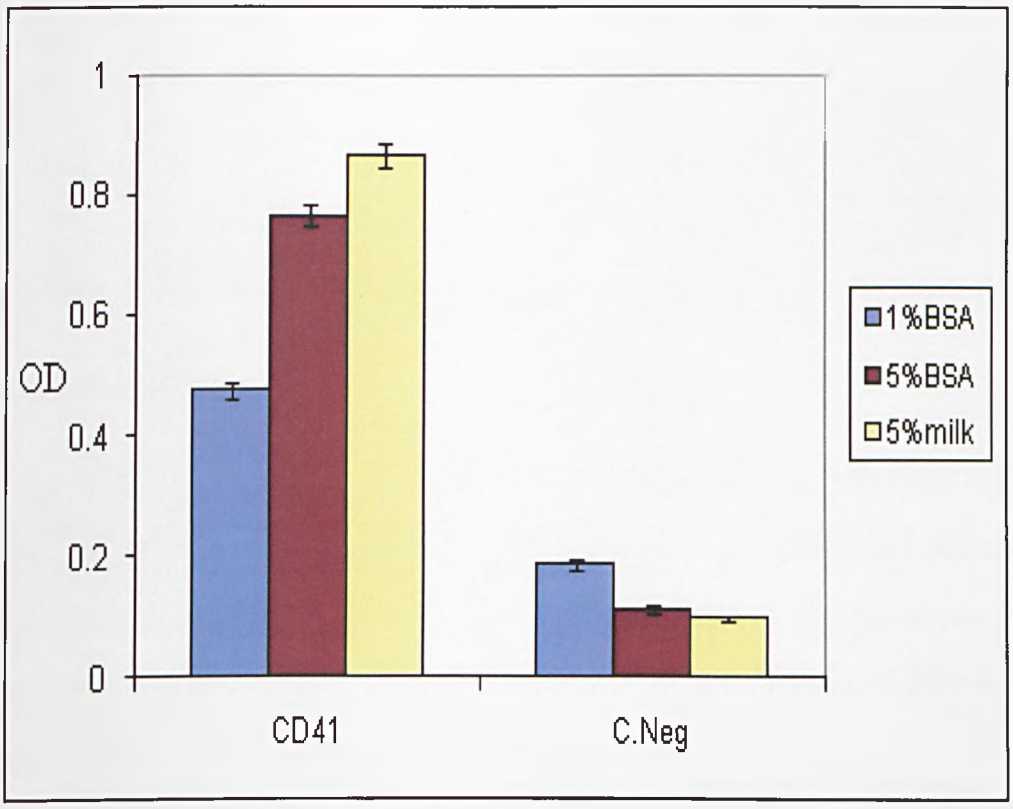


Figure III–1 ELISA results for three different blocking buffers, BSA 5%, BSA 1% and dried milk 5% in PBS . Bars represent the anti-CD41 reactivity against CD41 on the membrane platelet. Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment. The negative control negative (C.Neg) was a 1:1 mixture of PBS and milk instead of anti-CD41.

III–3 Platelet Protein Isolation and Detection

III–3.1 Protein Assay

Platelet lysate was isolated from 60 ml of anti-coagulated normal blood and assayed for protein content after concentration with a Centriplus centrifugal filter device with molecular weight cut-off of 10 kD. The protein content of the platelet lysate was measured by the modified Bradford assay (Bradford 1976). BSA at an initial concentration of 1.6 mg/ml was used to produce a standard curve by serial dilution (section II.2).

The standard curve obtained from different dilutions of BSA is shown in Fig. III–2. The Triton X-100 supernatant contained 1400 µg/ml of platelet protein after concentration. It was used to investigate the effect of varying concentrations of platelet lysate in an ELISA assay (Fig. III–2). The platelet lysate was prepared from the different healthy volunteers, and measured, which was used at a concentration of 200 µg/ml in all subsequent experiments (section II.4).

Figure III–2 Standard curve obtained from different dilutions of BSA protein

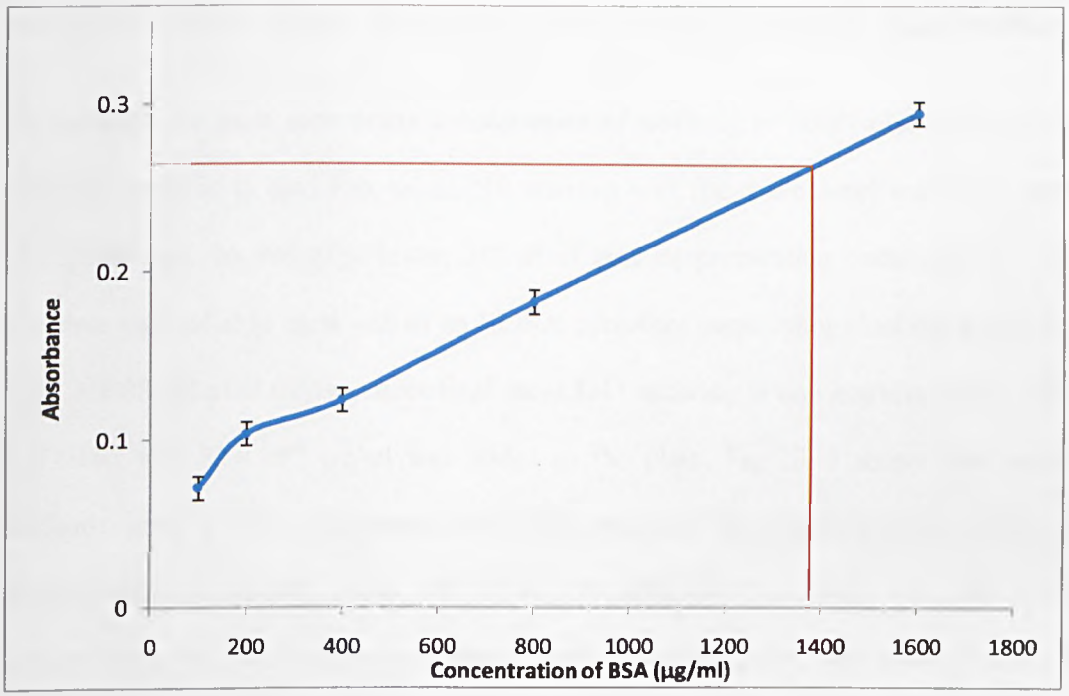


Figure III–2 The standard curve for absorbance BSA protein concentration used to determine the protein concentration of the platelet lysate. Two wells were used for each determination.

III–3.2 Detection of Platelet Glycoproteins using Different Antibody Concentrations

To establish the most appropriate concentration of antibody to detect platelet glycoproteins with the potential to bind Fab, an ELISA analysis with the monoclonal anti-CD41 antibody was performed. In this experiment, 100 µl of platelet preparation containing 10^7 washed platelets was added to each well of an ELISA microtitre plate. After blocking with 5% dried milk in PBS, 50 µl of mouse monoclonal anti-CD41 antibody in concentrations over the range 1.0×10^{-2} to 6.25×10^{-4} µg/ml was added to the plate. Fig. III–3 shows that anti-CD41 antibody gave a high absorbance at the first dilution, but absorbance decreased as the concentration decreased. Thus 10^{-2} µg/ml of anti-CD41 antibody was the optimum concentration and this was the concentration used in all subsequent experiments (section II.5).

Figure III–3 The effect of using different anti-CD41 antibody concentrations for the detection of platelet glycoprotein

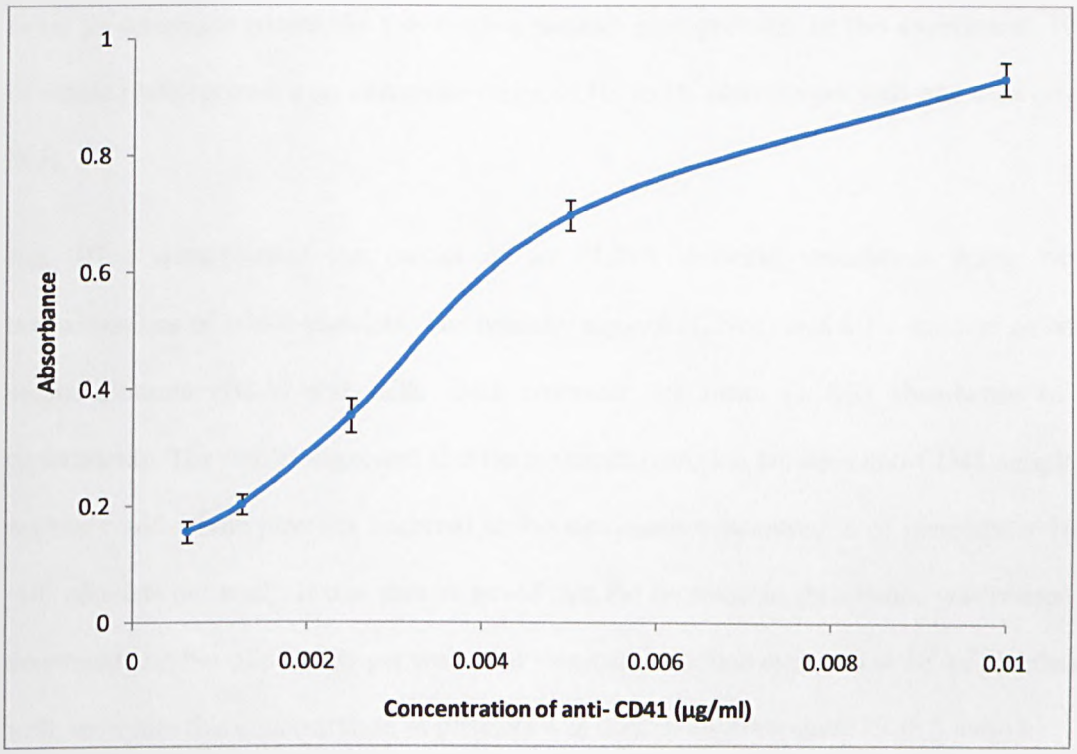


Figure III–3 Effect of the concentration of monoclonal anti-CD41 antibody on the absorbance observed in an ELISA. Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.

III–3.3 Optimum Concentration of Whole Platelets for Use in the ELISA

ELISA was performed to identify the optimum concentration of whole platelets to use in order to determine potentially Fab-binding platelet glycoproteins. In this experiment, 100 µl of whole platelets over a concentration range of 10^7 to 10^4 platelets per well was used (section II.5).

Fig. III–4 demonstrates the results of an ELISA showing absorbance using varying concentrations of whole platelets. The negative control (C.Neg) was a 1:1 mixture of bovine serum albumin (BSA) and milk. Data represent the mean (± SE) absorbance of two experiments. The results suggested that the maximum reaction between anti-CD41 antiplatelet antibody and whole platelets occurred at the maximum concentration of platelets at 10^8/ml (10^7 platelets per well). It was also observed that the decrease in absorbance was related to a decreased number of platelets per well. The maximum reaction occurred at 10^7 of platelets per well, therefore this concentration of platelets was used in all subsequent ELISA assays.

Figure III–4 Binding curve for different numbers of whole platelets against anti-CD41 antibody

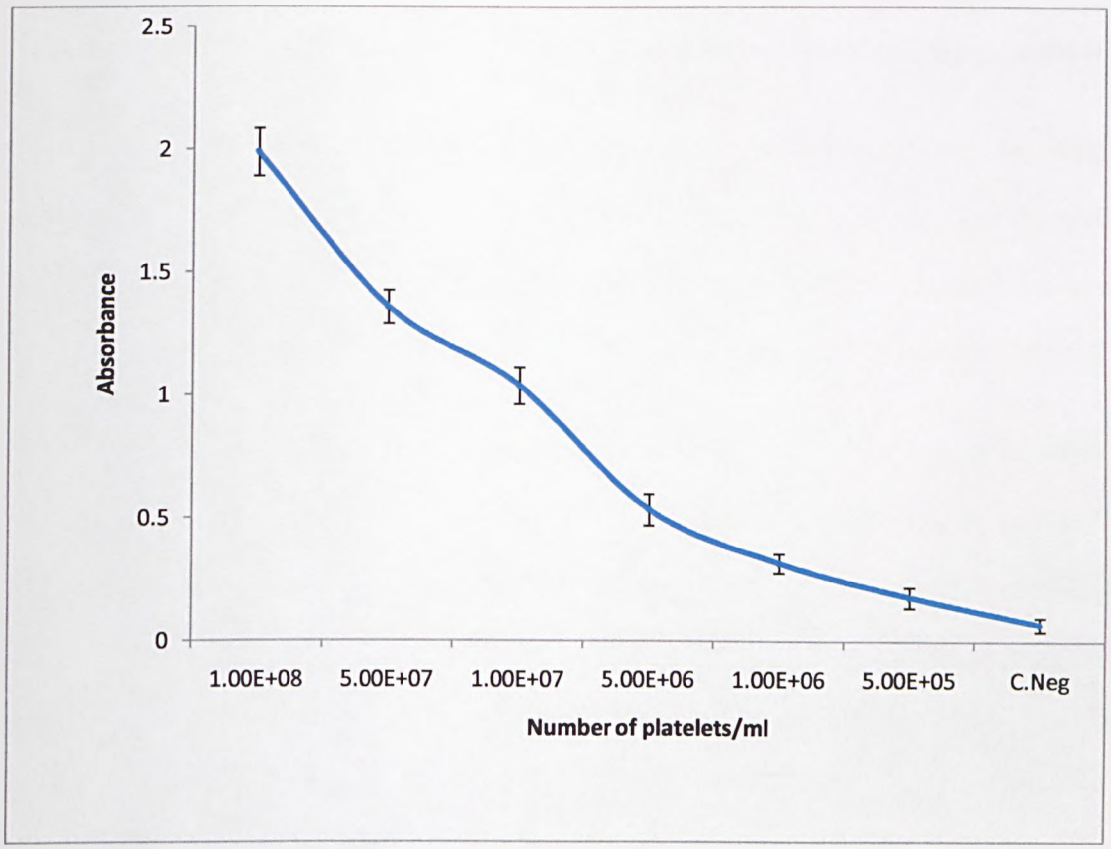


Figure III–4 ELISA showing absorbance for different concentrations of whole platelets. The negative control (C.Neg) was a 1:1 mixture of bovine serum albumin (BSA) and milk. Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.

III–3.4 Optimum Concentration of Platelet Lysate for Detecting Anti-CD41 Antibody

ELISA was used to determine the optimum concentration of platelet lysate to be used for detecting anti-CD41. In this case, 100 µl of platelet lysate over the concentration range of 100–800 µg/ml was loaded in each well. Then 100 µl of the anti-CD41 antibody, diluted 1 in 100 with 5% dried milk in PBS was added. The absorbance was read at 450 nm(section II.5).

Fig. III–5 shows the results of this ELISA. In order to plot the graph, the concentration of platelet lysate was chosen as the independent variable, represented on the x-axis, and absorbance was shown on the y-axis as the dependent variable. It shows that decreasing the concentration of platelet lysate per well decreases the amount of binding of anti-CD41 antibody. The highest concentration of platelet lysate gave the highest absorbance. The optimum concentration of platelet lysate was detected at a concentration of 200 µg/ml which it is the same amount of protein of 10^7 whole platelet per well (Kelton 1983) ,and it is used in other studies.

Figure III–5 Th e effect of different concentrations of platelet lysate on the binding of anti- CD41 antibody

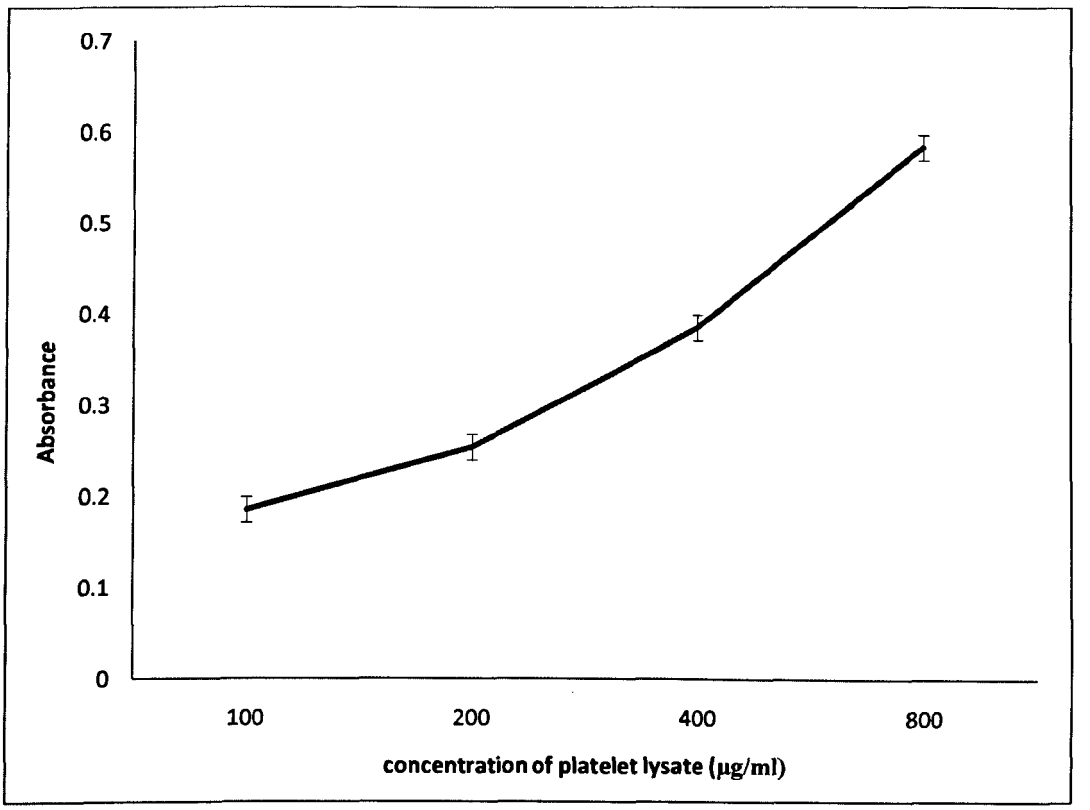


Figure III–5 The effect of varying platelet lysate concentration on the binding of anti-CD41 antibodies, as shown by the absorbance. In order to plot the graph, concentration of platelet lysate has been chosen as the independent variable represented on the x-axis and absorbance has been shown on the y-axis as the dependent variable. The experiment was performed twice, two wells were used for each experiment.

III–3.5 Binding of Antibodies to Platelet CD41 on Whole Platelets and Platelet Lysate

To determine whether the whole platelet preparation or platelet lysate functions better as a substrate, for use in future assays, an ELISA analysis of the platelet glycoprotein IIb/IIIa complex was undertaken using anti-CD41 antibodies.

Fig. III–6 shows the ELISA analysis of anti-CD41 antibody reactions to 10^7 whole platelets per well and 100 µl per well of platelet membrane lysate at a concentration of 200 µg/ml, it is the same amount of protein of 10^7 whole platelets per well (Kelton 1983). There was a significant reaction of the antibody against CD41 in the platelet lysate preparation, with an even stronger reaction occurring in the whole platelet reaction.

Figure III–6 A comparison of the binding of antibodies to platelet CD41 on whole platelets and platelet lysate

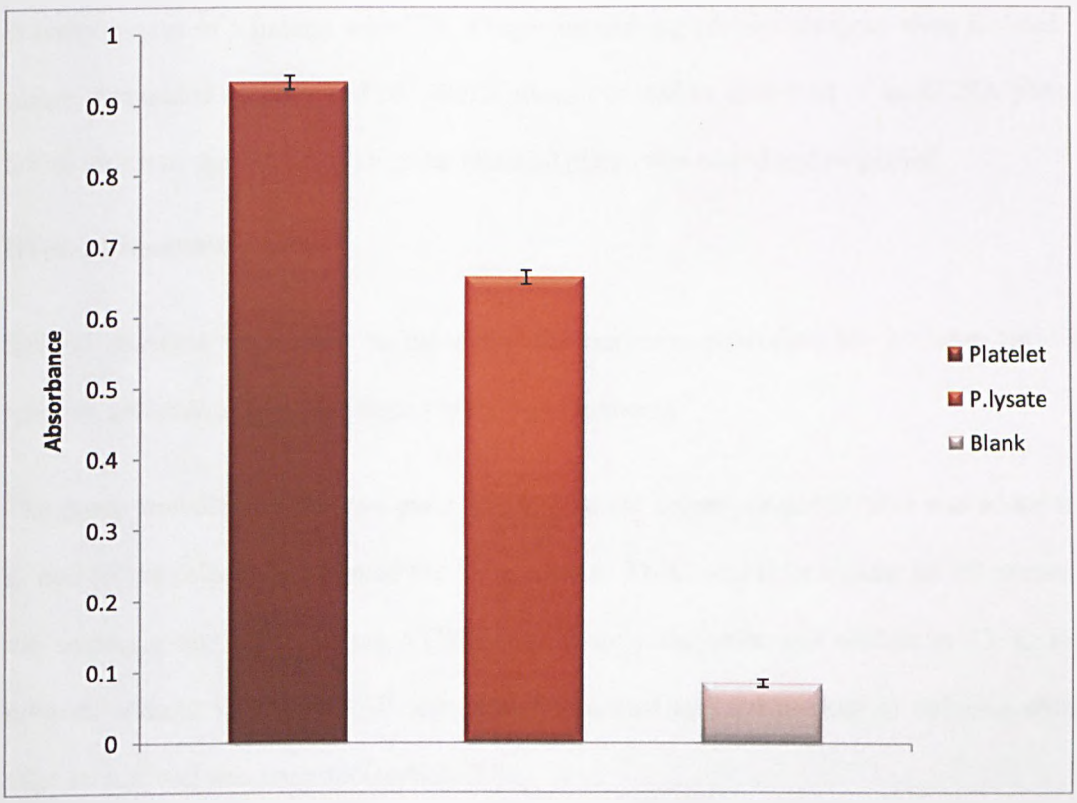


Figure III–6 ELISA comparing the binding of monoclonal anti-CD41 to whole platelets and platelet lysate. Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment. Blocking buffer (5% dried milk in PBS) was used as a blank for control.

III–4 Isolation of Antiplatelet Fab Fragments

The Fab library was constructed at the University of Sheffield from the splenic B lymphocytes of a patient with ITP. Phage-recognising platelet antigens were isolated. The phage was added to 100 µl of 10^7 whole platelet coated to each well of an ELISA plate and left to attach to the platelets. Then the attached phage was eluted and amplified.

III–4.1 Biopanning

Several attempts were made to determine the optimum procedure for isolating anti-CD41 reactive antibodies from the phage library by biopanning.

The group was divided into two parts. In the first, the helper phage (VCSM) was added to the *E. coli* (K12) cells and incubated for 15 minutes at 37 °C, and then shaken for 60 minutes. In the second group, after adding VCSM immediately the cells was shaken at 37 °C for 60 minutes without incubating. All cells were biopanned and the number of colonies obtained after each round was recorded(section II.8).

The number of washes of the microwells was also studied. Wells were washed either one or five times each with a solution of 0.05% Tween 20 in PBS.

These experimental conditions are shown in Table III–1. The results suggest that incubation of the cells with addition of helper phage, followed by five times washes the microplate are suitable conditions for the remaining experiments.

Table III–1 The number of the colonies in the biopanning on different condition of incubating

	Incubation	Number washes	Number of colonies after each round of Biopanning				
			R1	R2	R3	R4	R5
Gro A	**Incubate**	**1**	**3**	**18**	**225**	**>1000**	**>1000**
	Incubate	**5**	**3**	**3**	**12**	**65**	**237**
Gro B	**None**	**1**	**3**	**0**	**0**	**0**	**0**
	None	**5**	**5**	**0**	**0**	**0**	**0**

Table III–1 Effect of incubation, shaking and number of microwell washes on biopanning of E. coli preparations. Cells were either incubated for 15 minutes at 37 °C, followed by shaking 60 minutes, and other group was shaken for 60 minutes at 37 °C without incubation.

During five rounds of biopanning, the enriched phage was monitored by counting the phage-infected colonies at the end of each round. Fig. III–7 shows the number of colonies isolated after each round. The number of colonies increased after each round, suggesting that the concentration of the phage potentially bearing anti-platelet Fab increased after each round.

Figure III–7 Number of colonies obtained from consecutive rounds of the biopanning against whole platelet

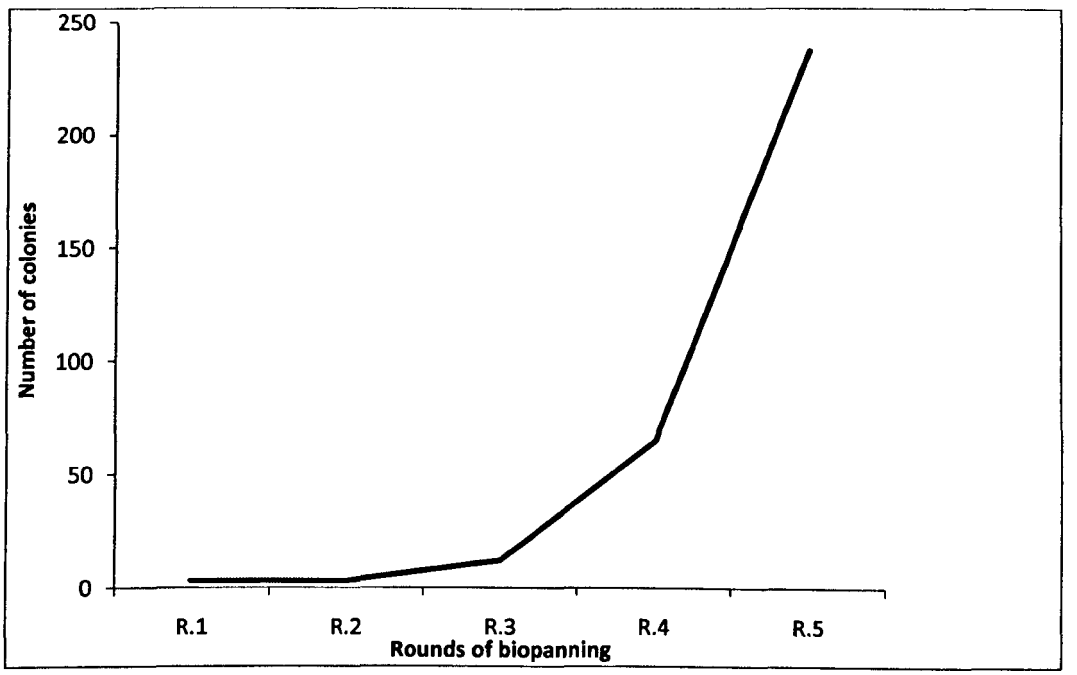


Figure III–7 Number of enriched potentially anti-platelet Fab bearing phage (number of colonies) after each round of biopanning.

The specificity of the isolated phage of each round was also examined for reactivity with platelet proteins using whole-platelet ELISA (section II.11.2). As can be seen from the graph (Fig. III–8), the absorbance was increased after each round of biopanning, indicating that the concentration of the phage potentially bearing anti-platelet Fab increased after every round.

Figure III–8 The specificity of the isolated phage of each round against whole-platelet Ag

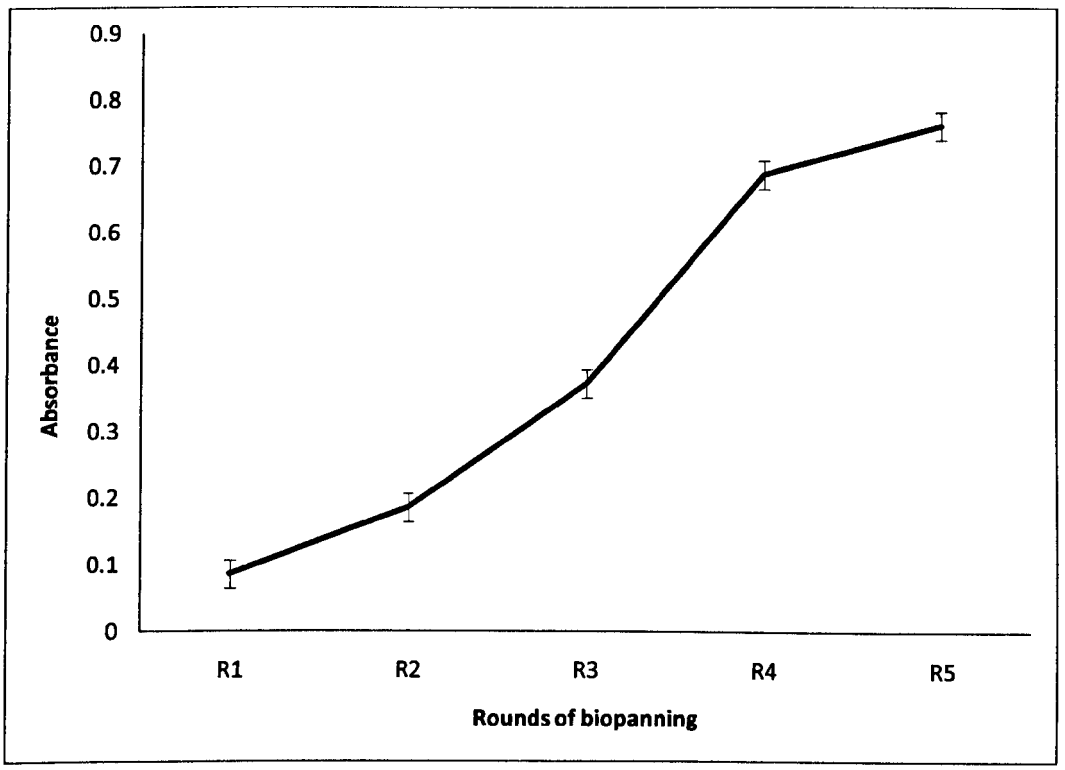


Figure III–8 Reactivity (absorbance) of the isolated phage against platelet Ag after each round of biopanning using whole-platelet ELISA. In this experiment, 100 µl of whole platelets (10^7 /well) was used (section II.7).

Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.

III–4.2 Specificity of the Isolated Phage

A hundred individual colonies isolated from the phage pool obtained after five rounds of panning were examined for reactivity against platelet proteins using the whole platelet ELISA. These colonies of phage isolated from the fifth round of panning were randomly selected and 10^9 colony forming units (c.f.u.) of phage were added to each well. The anti-phage antibody was employed to detect the binding of this phage to platelets (section II.11.4). Of these hundred colonies, just twelve reacted with whole platelets. These were S1, S2, S3, S5, S7, S8, S9, S11, S12, S14, S18 and S20.

Fig. III–9 shows that the amount of reactivity of the 12 colonies for binding with whole platelets. M13 (helper phage) was used as a negative control and anti-CD41 as a positive control. PBS was the blank. Most of the colonies had absorbance between 0.5 and 0.6, with the highest reactivities against whole platelets being seen around absorbance of 0.6.

Figure III–9 Reactivity of the isolated phages from 12 individual colonies after five rounds biopanning against platelet proteins using whole platelet ELISA.

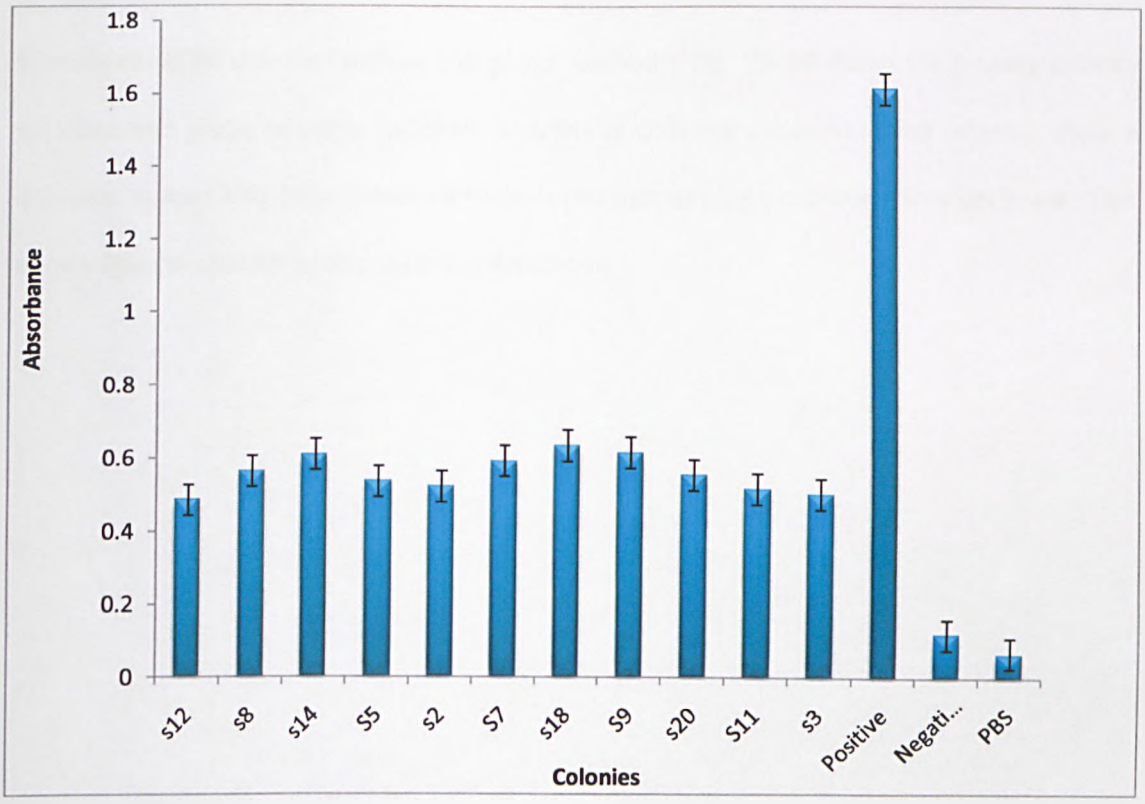


Figure III–9 Whole-platelet binding activity (absorbance) of 12 colonies isolated after five rounds of panning, plus a negative control (M13), a positive control (anti-CD41) and a blank (PBS). In this experiment, 100 µl of whole platelets (10^7 /well) was used (section II.7).

Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.

III–4.3 Titration of Isolated Phage Against Whole Platelet

Two of the high-reacting isolated phage colonies, S7 and S9, were used to test reactivity against whole platelets in a titration experiment. They were serially diluted from 1 in 100 to 1 in 800, then 100 µl of each dilution was added to the wells so that the degree of phage-binding could be detected with an anti-phage antibody. Fig. III–10 shows the binding activity of these two phage colonies to whole platelets at different dilutions. Both colonies show a decrease in reactivity (absorbance) for whole platelets as phage concentrations get lower. This shows that the activity is concentration dependent.

Figure III–10 Reactivity of two isolated phage against platelet protein at different concentration using whole platelet ELISA

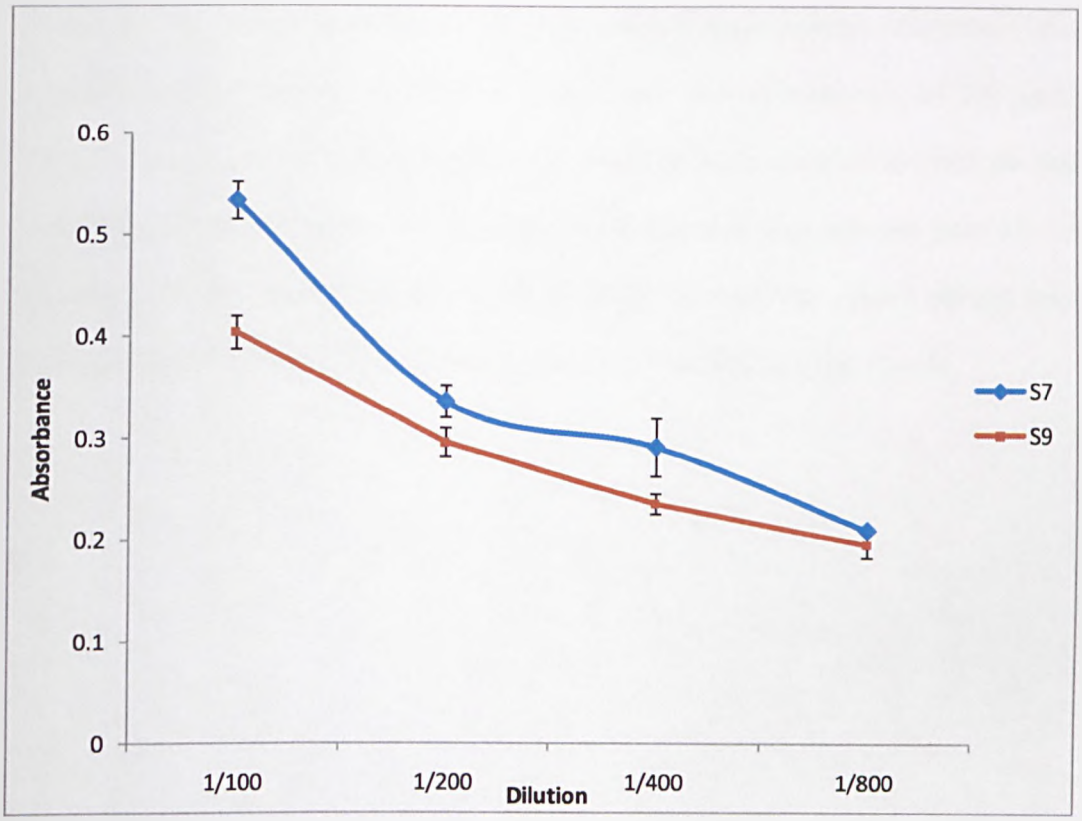


Figure III–10 Effect of dilution of phage from two reactive colonies (S7 and S9) on binding (absorbance) to whole platelets. Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.

III–4.4 Binding of Isolated Phage from Biopanning Against Platelet Lysate

The twelve colonies that showed strong reactions against whole platelets were analysed to determine their degree of binding to the lysate rather than the platelet. Microtitre plates were coated with 100 µl of the platelet membrane lysate at a concentration of 200 µg/ml, and 100 µl of phage was added to each well. Anti-phage antibody was used to detect the degree of binding to the platelet lysate. The results showed that only four colonies from 12 colonies, which reacted with whole platelets, showed substantial reactivity against platelet lysate. S7 showed highest binding to both platelet lysate and whole platelet (Fig. III–11).

Figure: III–11 Reactivity of isolated phage from biopanning against platelet protein using platelet lysate ELISA

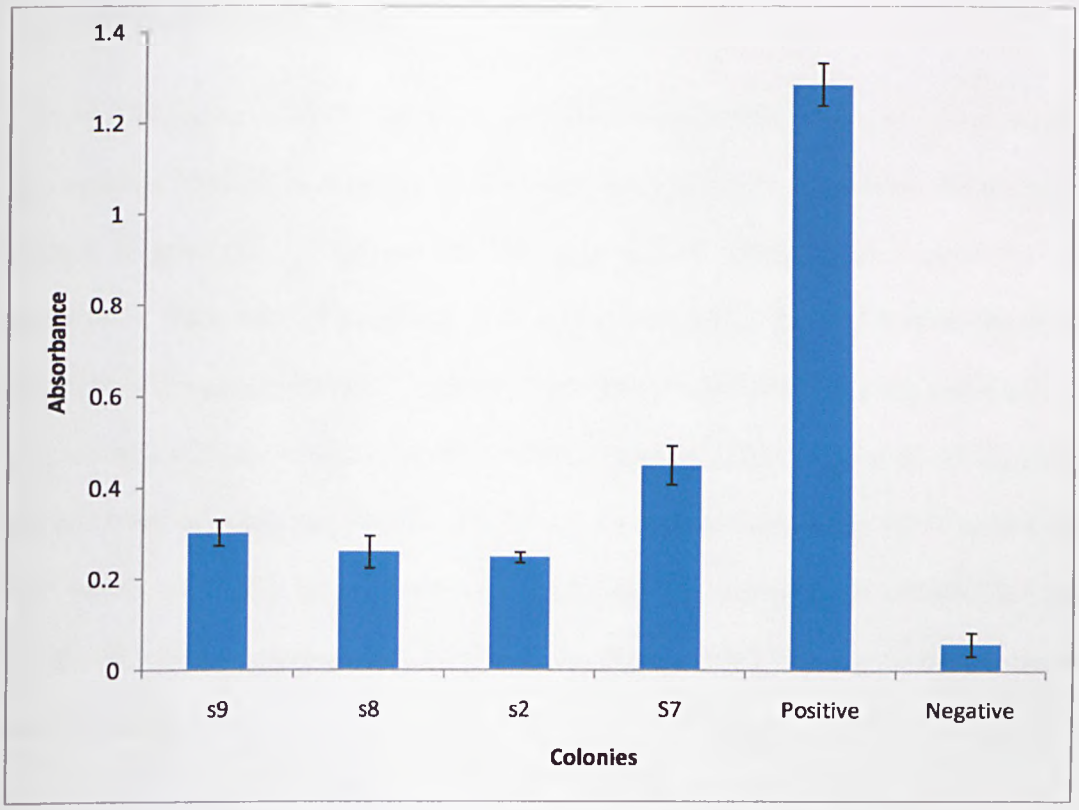


Figure: III–11 Reactivity (absorbance) against platelet lysate of phages from four colonies isolated by biopanning. In this experiment, 100 µl of platelet protein (200 µg/ml) was used (section II.5). Anti-CD41 and M13 were used as control positive and negative respectively.

Data represent the mean (±SE) absorbance of three experiments. 2 wells were used for each experiment.

III–4.5 Reactivity of Phage Isolated from ITP Library against Purified Platelet Glycoprotein IIb/IIIa Complex

The most likely candidate for the antigen recognised by the Fab bearing phages is the platelet glycoprotein GP IIb/IIIa complex. The colonies that showed strong positive reactions against platelet antigens (S2, S7, S8 and S9) were also used to determine their specificity against GP IIb/IIIa. Each well of microtitre plate was coated with 100 µl of purified glycoprotein GP IIb/IIIa at a concentration of 2 µg/ml. Then 100 µl of 10^9 of Fab-bearing phage was added to each well with anti-phage antibody to detect their specificity. Figure III–12 demonstrates the reactivity of phage colonies S2, S7, S8 and S9 against the purified GP IIb/IIIa complex. The results show that the selected colonies reacted strongly with the GP IIb/IIIa complex compared with the negative control (M13 helper phage), with S7 having the highest reactivity, and S2 the least.

Figure III–12 Reactivity of isolated phage from biopanning against purified platelet glycoprotein IIb/IIIa complex using ELISA assay

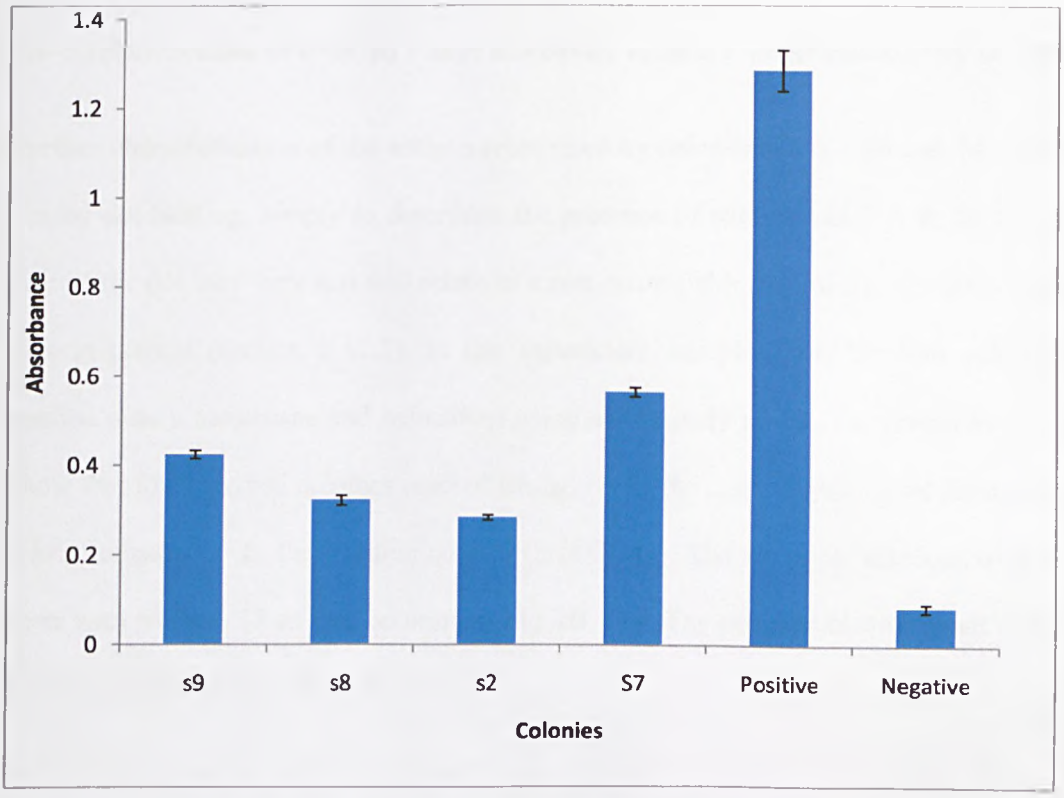


Figure III–*12* Reactivity (absorbance) of antiplatelet Fab antibody against purified platelet glycoprotein IIb/IIIa complex in four phage colonies. In this experiment, 100 µl of purified IIb/IIIa complex (2 µg/ml) was used (section II.5).

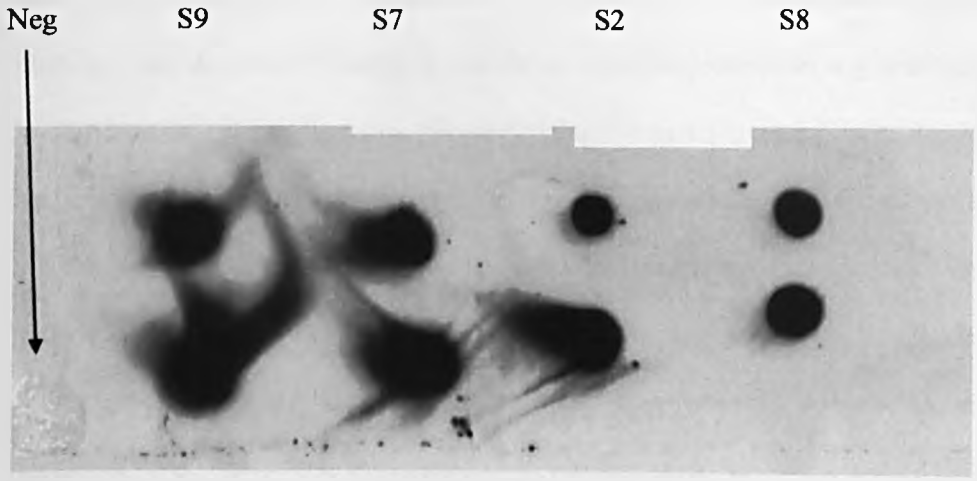
Comparison is made with a positive (anti-CD41) and negative control (M13). These data represents the mean absorbance (± SE) from two experiments. Three wells were used for each determination in each experiment.

III–5 Identification of Isolated Phage Reactivity against Platelet Proteins by Dot Blotting

Further characterisation of the antigen recognised by colonies S2, S7, S8 and S9 was carried out by dot blotting, simply to determine the presence of anti-platelet Fab in the phage. The size of the dot may vary and will relate in a non-quantifiable way to the amount of the target protein present (section II.12.1). In this experiment, samples from the four colonies were spotted onto a membrane and hybridised using an antibody probe .The results in Fig. III–13 show that four selected colonies reacted strongly with the platelet lysate used for dot blotting, when compared with the positive control (anti-CD41). The strongest reactions overall were seen with S9 and S7 as can be seen in Fig. III–13A The positive control result using anti-CD41 is shown in Fig. III–13B.

Figure III–13 Reactivity of isolated phage from biopanning against platelet protein using dot blotting

A: S9, S7, S8, S2



B: CD-41

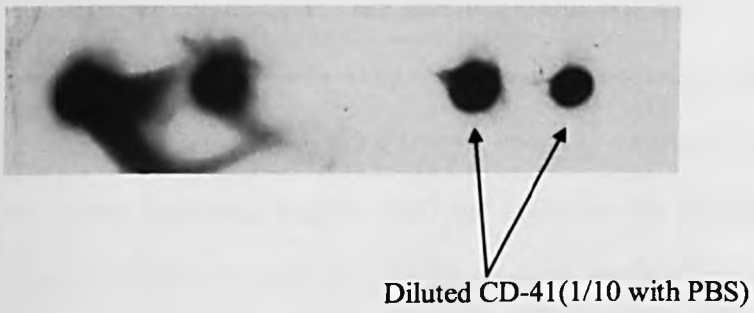


Figure III–13 Dot blotting of human platelet lysate reacting against four Fab-bearing phage colonies (S2, S7, S8 and S9) compared with the positive (anti-CD41), and Negative (5% BSA) controls.

III–6 Identification of Isolated Phage Reactivity Against Platelet Proteins by Western Blotting

The antigen recognised by colonies S2, S7, S8 and S9 was further characterised by western blotting. This analytical technique can detect specific proteins in a given sample of tissue homogenate or extract, by using gel electrophoresis to separate native or denatured proteins according to the length of the polypeptide. The proteins are then transferred to a nitrocellulose membrane or polyvinylidene fluoride (PVDF) membrane, where they are probed by antibodies specific to the target protein. In this experiment, 20 µl of platelet lysate at a concentration of 200 µg/ml was loaded onto an 8% polyacrylamide gel. Electrophoresis was performed and it was then transferred onto nitrocellulose paper (section II.12.2). Lanes S2, S7, S8, and S9 are the anti-platelet Fab bearing phage which reacts with platelet lysate.Lane N is a negative control (M13) and lane P is positive (GPIIIa) control. At room temperature of 15–22 °C) three bands with different molecular weights were identified by the S2, S7, and S8 colonies of isolated Fab-bearing phages, and only one band was detected for the phage of the S9 colony. Molecular weights 92kD and 116kD are the same GpIIIa, and GPIIb respectively. The minor bands are more likely break down of platelet glycoprotein.

These results are shown in Fig. III–14

Figure III–14 Reactivity of isolated phage from biopanning against platelet protein using western blotting developed by ECL.

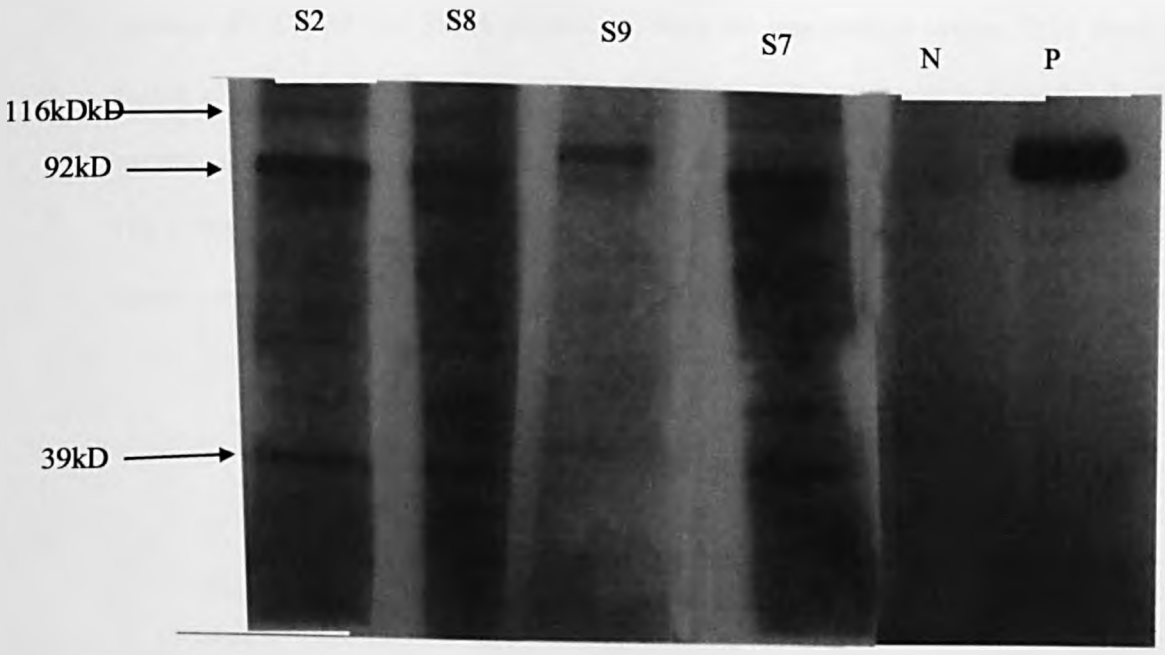


Figure III–14 Western blotting of non-reduced human platelet lysate reacting against four Fab-bearing phage colonies (S2, S7, S8, S9) along with negative (M13 helper phage) and positive (anti-CD41) controls. The first four lanes are the platelet protein bands for the four colonies. Lane N is the negative control and lane P is the positive control.

III-7 DNA gel agarose

In order to determine that heavy and light chains were present in the phage, DNA of the four phage colonies with stronger reactions to platelet protein were analysed. Again these were the colonies S2, S7, S8 and S9. A plasmid isolation kit was used to isolate DNA from phage grown in Luria broth. The purified DNA from each phage colony was cut with a restriction enzyme and loaded onto an agarose gel before undergoing electrophoresis (section II.10.3). The results demonstrated the presence of a DNA band of 5.4 kbp (base pairs) in size. The figure shows the presence of library phage DNA of the correct molecular weight (Fig. III–15).

Figure III-15 Purity of phage DNA

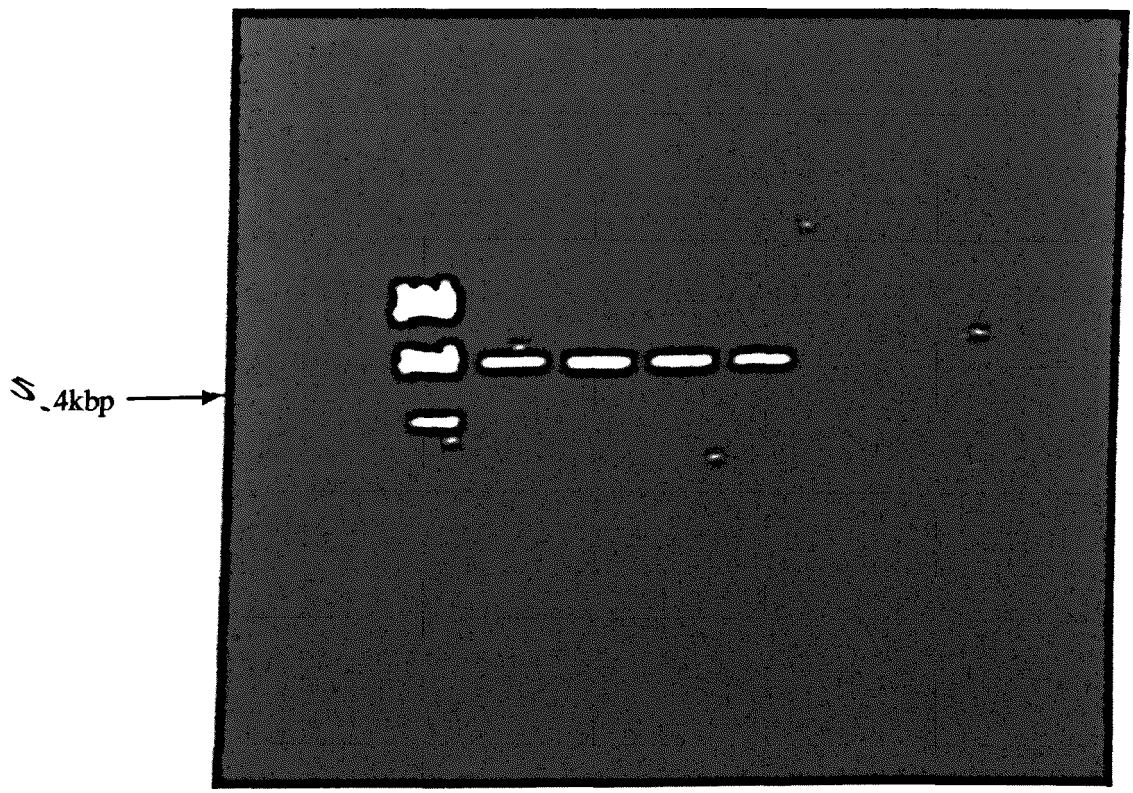


Figure III-15 Purified DNA of the four the phage run on a 1% agarose gel. The four indicated bands (S2, S7, S8, and S9) co-migrated with the 5.4kbp DNA molecular size marker.

SECTION IV: DISCUSSION

The thrombocytopenia in ITP characterised by the destruction of platelets in the circulation by antibodies. These antiplatelet antibodies react with various autoepitopes on the glycoprotein complexes, located on the external membrane of platelets, which are the focus of the present study.

IV–1 ELISA of antiplatelet Antibodies

In the initial stages of the study, a quick and sensitive ELISA assay was developed for analysing whole washed platelets and frozen platelet lysate from healthy volunteers, in order to identify and quantify the binding of antiplatelet antibodies relevant to the disease ITP. All the glycoprotein subunits on platelets can act as antigens to autoantibodies, but the complexes commonly affected in chronic forms of ITP are the highly immunogenic GP IIb/IIIa and GP Ib/IX complexes (Van Leeuwen 1982; Beardsley 1984; Woods 1984; Yanabu 1991). Around 75% of antiplatelet antibodies in ITP are against antigens on the GP Ib–IX complex, and anti-GP IIb/IIIa autoantibodies occur in approximately 15% of ITP patients (McMillan 2000). It was expected during this study that any antibody reactivity would be directed against one of these types of glycoprotein receptors.

The initial ELISA studies focused on the adhesive protein receptor complex known as GPIIb/IIIa, which is associated with two antigens, namely the CD41 antigen and the CD61 antigen. The GP IIb complex is the CD41 antigen and the GP IIIa complex is the CD61. A reaction was only obtained for anti-CD41, so the subsequent experiments were based on the

use of anti-CD41 as control positive. Throughout this discussion, antibodies against the GPIIb complex will be referred to as anti-CD41.

The GP IIb/IIIa complex is of particular significance in ITP as it is also the fibrinogen receptor, which is essential for maintaining normal haemostatic functioning of the body (Beutler 1995; Stiene-Martin 1998). Circulating platelet counts decrease through autoantibody-mediated destruction when the glycoprotein receptors are seen as antigens, and disruption of this complex also alters the platelet response to injury. The overall result is the development of bleeding disorders such as purpura and prolonged bleeding time, as seen in ITP.

Having established the presence of glycoprotein antigens in the human platelet samples, various parameters were investigated for optimum performance in the ELISA experiments. These included the choice of blocking buffer. Different kinds of blocking buffers were investigated to find the most appropriate one for use in subsequent ELISAs experiments. The buffer which gave greatest absorbance difference between positive and negative control was 5% dried milk in PBS (Fig. III–1).

The platelets from the healthy volunteers were prepared in two ways – as a whole platelet preparation or as a lysed platelet preparation. The amount of protein contained in the lysate was determined using a modified Bradford assay (Bradford 1976). Every ml of lysate was found to contain 1400 μg protein (Fig. III–2). The value of 200 μg protein per ml was used in all subsequent experiments, which it is the same amount of protein of 10^7 whole platelet per

well (Kelton 1983). This protein would have included antigens such as CD41 and other glycoproteins, as well as other non-glycoprotein proteins.

IV–2 ELISA of Antiplatelet Fab Antibody Fragments

ELISA was also used to detect potentially Fab-binding platelet glycoproteins phages. This is because antibody binding to glycoprotein complexes in platelets can be Fab-mediated (Stricker 1986), whereby the Fab-binding part of the glycoprotein binds to the Fab region of the paratope on the antibody.

To detect these platelet Fab-binding glycoproteins, it was necessary to quantify the optimum concentrations of anti-CD41 antibody to use in the ELISA experiments. The optimum concentration was therefore determined using mouse monoclonal anti-CD41 antibody and washed whole platelets (Fig. III–3). It was found to be 10^{-2} µg/ml.

Then the optimum concentration of whole platelets to use in ELISA experiments was found. Whole platelet preparations in a range of concentrations were tested against anti-CD41 antiplatelet antibody, and a maximum reaction was observed with 10^{7} platelets per well. This whole-platelet concentration was used in all subsequent ELISA assays.

The optimum concentration of platelet lysate for detecting anti-CD41 was determined to be 1400 µg/ml (Fig. III–5) that it was used at 200 µg/ml throughout the study.

These optimum concentrations of whole platelets and lysate were used to determine whether whole platelets or lysate were more suitable as a substrate in the binding assays of antibodies and platelet antigens. Whole platelets were expected to adsorb more antibodies against

glycoprotein complexes, not least because antiplatelet antibodies can only attach to cation-dependent epitopes on the GP IIIa component if the complex is conformationally intact (McMillan 2000), or because the complex on the platelet surface may undergo a conformational change that causes it to be displayed differently on activated platelets and express new epitopes (Wagner 1996).

In the ELISA analysis of the platelet glycoprotein IIb/IIIa complex, using monoclonal anti-CD41 antibodies, the anti-CD41 antibody reacted most strongly against CD41 antigen in the whole platelet preparation, as expected, compared with the antigen in the lysate preparation (Figure III–6). The reaction of the lysed platelets was only 70% of that seen with the whole platelet preparation. There may be several reasons for this. First, the amount of CD41 antigen may have been greater in the whole platelet preparation because there may have been different amounts of protein in the two kinds of samples. Only the protein content in the lysed preparation was determined in this experiment. The protein content of the whole platelet sample may have been different, which meant the two samples could not be directly compared and no assumptions could be made about the amount of CD41 antigen they contained. As a consequence, any differences in the reactivity of the samples in this study had to be treated with caution.

Second, the differences obtained with the different preparations might have arisen because of changes occurring during the preparation of the lysed platelets. Lysis may release non-membrane proteins and expose new antigenic epitopes. The glycoprotein complexes may remains intact on the surface of the platelet, and some become detached from the platelet

membranes and undergo some conformational change. Evidence suggests that some complexes are better recognised by antibody when they are still *in situ* on the surface of the platelet.(McMillan 2000), for example, showed that antiplatelet antibodies only attach to cation-dependent epitopes on the GP IIIa component when the whole GP IIb/IIIa complex is conformationally intact.

IV–3 Isolation of Antiplatelet Fab Fragments

Antiplatelet Fab fragments specific for the glycoprotein complex were isolated from a phage library by a procedure known as biopanning, which involves several steps. The biopanning experiments used whole platelets, rather than lysed platelets, largely because the response of autoantibody in ITP is against several glycoproteins or antigens on the surface of the platelet (Smith 1994).

First, a phage display library was prepared from a Fab-expressing phagemid library of human immunologlobulin light and heavy antibody chains on the V domain, constructed from the mRNA from the splenic B lymphocytes of a patient with ITP, using cDNA encoding for immunoglobulin mostly IgG. IgG is associated with the chronic form of ITP (Shulman 1965; Shulman 1965; McMillan 1974; Schwartz 2007), and IgG-specific autoantibodies can be detected in as many as 60% of ITP patients (Coopamah 2003)with levels of platelet-binding IgG being shown to increase (McMillan 1974; Semple 1998).

In this study, PCR-amplified gene segments for the antibody domains which were cloned into PAK100 vectors (Krebber 1997). The desired gene segments were inserted into the phagemid of the *Escherichia coli* and the gene product was displayed on the surface of the

bacteriophage. During the capturing step, the phage library was conjugated to the desired target, so that only specific peptides presented by bacteriophage were bound to the target. The bacteria that took up copies of the plasmid became resistant to certain antibiotics, and on exposure to a particular antibiotic, the resistant bacteria survived. These bacteria were grown, harvested and lysed to isolate the plasmid of interest. Any phages that did not bind to the solid surface are washed away, leaving phages with strong binding affinity. The more times the preparation was washed after each round of panning resulted in the removal of more unbound phage, without significant loss of specifically bound phage.

The library underwent sequential panning with whole platelets from the healthy volunteers, and resulted in the isolation of glycoproteins that recognised the phage specifically. Phage-recognising platelet antigens that bound the Fab fragments were isolated and the attached phage was recovered and amplified and used to infect more *E. coli*. The more times this was done, the stronger the affinity of the binding peptides to the target.

In order to isolate maximum numbers of potentially anti-platelet antibodies, the biopanning procedure was carried out under different conditions, with either incubation or shaking immediately after adding VCSM helper phage, and with different numbers of washes after each round of biopanning. This study revealed that the number of colonies obtained was maximal with incubation rather than shaking and with five washes after each round of biopanning (Table IV–1).

Five rounds of biopanning were carried out. Each round produced higher numbers of enriched antiplatelet antigen-specific Fab-bearing phage. The amount was quantified as colony-

forming units (c.f.u.) as shown in Fig. III–7. The first round of panning produced only three clones from a 10^9 c.f.u./ml phage suspension. After five rounds there were 237 colonies. The quantity of starting phage was not significant.

It was expected that these increasingly large numbers colony-forming units would have increased levels of anti- Fab- bearing phage. The specificity of the enriched phage in each round of panning was therefore tested for reactivity against platelet protein. ELISA was used to confirm that the amount of phage potentially bearing anti- Fab increased after each round of panning, reaching a maximum after the five rounds.

The reactivity of a number of colonies after five rounds of panning was investigated next. A hundred colonies were isolated from the phage pool, which randomly were selected. From each of these, 10^9 c.f.u. of the phage were added to a well with anti-phage antibody and examined for reactivity against platelet proteins using the whole-platelet ELISA. Twelve of the hundred colonies showed reactivity against whole platelets, as shown in Fig. III–9. All of the twelve reacting colonies showed a high level of reactivity with whole platelets compared to the negative control M13 phage (Fig. III–9). The same study showed that 23 out of 40 randomly selection colonies reacted with GPIIb/IIIa that their inhibition studies using murine mAb against various epitopes suggested that binding epitopes of phage antibodies were located within the GPIIb/IIIa complex. They used the phage display approach to isolate Fab antibody fragments recognizing native GPIIb/IIIa.A library from a human donor immunized with Rh^+ red blood cells was panned with freshly washed platelets.(Escher 1998).

IV.4 Identification the phage expressing anti platelet antibodies by platelet lysate

The phage colonies that showed a positive reaction against whole platelets were analysed to determine their binding to platelet lysate. The microtitre plates were coated with 100 µl of the platelet membrane lysate at a concentration of 200 µg/ml. The results showed that just the colonies S2, S7, S8, and S9, which strongly reacted with whole platelet, also reacted with platelet lysate when compared with negative control (M13), however these reactions are less than the reaction against whole platelets. S7 showed highest binding to both platelet lysate and whole platelet. After that, those colonies which showed a strongly positive reaction against platelet lysate were analyzed to determine their specificity against purified GPIIb/IIIa. Each well of microtitre plate was coated with 100 µl of purified glycoprotein IIb/IIIa at a concentration of 2 µg/ml. The result show that the selected colonies, which reacted strongly with whole platelets and platelet lysate, also reacted strongly with the GPIIb/IIIa complex.

IV–5 Identification of the reactivity of the isolated phage against platelet proteins by Dot blotting

Further characterisation of the proteins in the platelet lysate was performed by blotting techniques. Dot blotting was used to detect the presence of anti-platelet Fab in four high-reacting Fab-bearing phage colonies. All four colonies reacted strongly with the platelet proteins in the lysate, as shown in Fig. III–13. This semi-quantitative analysis revealed nothing other than the presence of the target molecule, giving no information about its properties, such as its molecular weight. Therefore western blotting was also performed on the phage colonies.

The ability of the same four Fab-bearing phage colonies to recognise platelet antigens was evaluated by quantifying the amount of protein through the non-reducing conditions of western blotting. This technique detects specific proteins in a sample using gel electrophoresis to separate them according to their molecular weight. In this experiment, samples from the four phage colonies were spotted onto a membrane and hybridised using an antibody probe. Three of the four colonies examined showed three bands. The bands represented proteins with several different molecular weights, including one of 92 kD. The fourth colony contained protein in just one band, and this band corresponded to a molecular weight of 92 kD. This means that all four colonies contained a protein with the same molecular weight as the positive control, that is 92 kD (Fig. III–14). The molecular weight of the glycoprotein GP IIIa subunit is between 88 and 95 kD (Kokawa 1993) so this experiment suggests the reactive protein in the four phage colonies was the GP IIIa glycoprotein subunit found in human platelets.

The final experiment to characterise the protein isolated from the phage library was a DNA gel agarose test. This was conducted on DNA isolated from the four high-reacting phage colonies. Each colony showed a DNA band that corresponded with the molecular size marker for 5.4 kbase pairs (Fig. III–15), and this suggested the presence of heavy and light antibody chains in the phage.

SECTION V: CONCLUSIONS

This study used a random selection of phage library colonies to produce Fab antiplatelet antibodies that showed reactivity for certain antigens on the surface of human platelets. This a phage library was derived from a patient with the autoimmune disease ITP. ELISA was used to show that the isolated Fab fragments reacted with antigens on the platelet surface. Future studies could be conducted to isolate additional Fab antibody fragments. Further analysis and modification of the fragments isolated in this study, and other Fab fragments isolated in the future, will improve our understanding of the platelet antigen–antibody interaction in the autoimmune disease of ITP.

There are numerous problems associated with the diagnosis of ITP, because the main features of the disease (thrombocytopenia and bleeding disorders) are not specific to this disease alone. Diagnosis must rely on laboratory findings (Brighton 1996) and the elimination of other causes of thrombocytopenia. Although some antiplatelet antibody assays have high specificity and accuracy, such as the MAIPA (Wadenvik 1998; Winiarski 1998), these assays are more suitable for monitoring disease severity rather than diagnosis. Any studies that further characterise the autoantibodies against platelet glycoproteins in ITP are likely to contribute to the body of evidence for making a more accurate and specifi clinical diagnosis, by helping to discriminate between the thrombocytopenia seen in ITP and thrombocytopenia that arises in other disease. Ultimately elucidating mechanisms behind the formation antigen–antibody complexes in ITP may offer new means of targeted therapy in the future.

VI. Future work

Further characterisation of the Fab can be undertaken by sequencing the DNA. This enables an understanding of the genetics of the expressed Fab repertoire (Chang 2001). It also gives a chance to study the mechanisms underlying the production of autoantibodies and structure motifs involved in antigen recognition. The DNA sequences from 4 colonies can show their sequences that these colonies have the same sequence and refer from one anti-platelet Fab or not. In one study DNA analysis from 23 phages which reacted against GPIIb/IIIa, identified only three Fabs (Escher 1998), which may suggest that patients with an immune response to GP IIIa may shows a small range of antibodies.

Additional isolation, analysis and modification of this antibody fragment holds potential for understanding key aspects of the platelet antigen-antibody interaction.

1-With manipulation of Fab bearing phage by further panning and selection of different colonies a more specific and higher affinity of Fab might be obtained.

2-By synthesising the peptides based on the RSD motif to analyses, their ability to inhibit platelet aggregation using aggregometer.

3-The RSD motif on CDR1 amino acid sequence can be used as a basis to develop therapeutic agents.

4-Site directed mutagenesis to analyse the role of individual amino acids in determining Fab binding to GPIIIa.

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